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MECHANISM OF METHOXYCHLOR TOXICITY IN MOUSE OVARIAN ANTRAL FOLLICLES

BY

MALLIKARJUNA SHIVAPURA BASAVARAJAPPA

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in VMS - Comparative Biosciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

Doctoral Committee:

Professor Jodi A. Flaws, Chair
Associate Professor Marie-Claude Hofmann
Professor Elizabeth H. Jeffery
Professor Romana A. Nowak
Assistant Professor Levent Dirikolu

ABSTRACT

1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane (methoxychlor; MXC) is an organochlorine pesticide used against pests and insects that attack crops, gardens, vegetables, pets, and livestock. MXC targets the ovary and its exposure has adverse effects on reproductive function in adult female mice causing persistent estrus, reduced fertility, and ovarian atrophy. MXC reduces fertility by increasing atresia of antral follicles and by decreasing numbers of healthy antral follicles in adult female mice. Further, MXC inhibits growth and induces atresia of mouse antral follicles in vitro. Little is known, however, about the mechanisms by which MXC causes slow growth and atresia of antral follicles. Hence, this dissertation work was designed to help us to better understand the mechanism of action of MXC in antral follicles by examining the effects of MXC on steroid levels, *Bcl2* factors and caspase activity, and by determining if MXC exerts toxicity through an AHR pathway. Since MXC is known to target antral follicles, the major producer of sex steroids in the ovary, the present study first tested the hypothesis that MXC decreases estradiol (E_2) levels by altering steroidogenic and metabolic enzymes in the antral follicles. Given that MXC induces atresia after long term exposure, in part, by increasing the pro-apoptotic factor *Bax* and decreasing the anti-apoptotic factor *Bcl2* in antral follicles, the present study also tested the hypothesis that MXC induces atresia at early time points and alters other pro-apoptotic (*Bok* and *Casp3*) and anti-apoptotic factors (*Bcl-xL*) in addition to *Bcl2* and *Bax*. In addition, the present study tested the hypothesis that MXC alters caspase activity in the follicles. Several studies indicate that many chemicals act through the aryl hydrocarbon receptor (AHR) pathway and one study has shown that MXC binds to the AHR in liver cells. Hence, the present work also tested the hypothesis that MXC binds to the AHR to inhibit follicle growth and

induce atresia of antral follicles. To test these hypotheses, antral follicles were isolated from ovaries of female wild-type (WT) or AHR knock-out (AHRKO) mice and cultured with either vehicle (dimethylsulfoxide; DMSO) or MXC. Follicle growth was measured every 24 h for 96 or 168 h. In addition, sex steroid hormone levels were measured using enzyme-linked immunosorbent assays (ELISA) and mRNA expression levels of steroidogenic enzymes, the E_2 metabolic enzyme *Cyp1b1*, and *Bcl2* related factors were measured using qPCR. Caspase activity and atresia were also measured in follicles. In granulosa cells, MXC binding to AHR was also measured using transfection experiments. The results indicate that MXC decreases most of steroidogenic enzymes, increases metabolic enzyme expression and this in turn leads to decreased sex steroid hormone levels. The results also indicate that, at 24 h, MXC increases *Bax* levels and does not affect *Bcl2* levels. This increases the *Bax/Bcl2* ratio, which in turn may increase the mitochondrial permeability leading to activation of caspase activities. Thus, these early changes in *Bax* expression and caspase may induce onset of morphological atresia beginning at 48 h. Further, the results indicate MXC binds to the AHR in granulosa cells and that MXC (10, 100 μg/ml) significantly inhibits follicular growth in WT antral follicles by 168 h. The results also indicate that MXC (10,100μg/ml) significantly induces atresia in WT antral follicles. Conversely, MXC (10 μg/ml) did not significantly inhibit growth or induce atresia in AHRKO follicles. These data indicate that AHR deletion protects antral follicles against MXC-induced growth inhibition and atresia. Thus, this study suggests that MXC acts through the AHR pathway to inhibit follicle growth and induce atresia in antral follicles of the ovary. Collectively, this dissertation work has shown that MXC exerts toxicity in antral follicles by inhibiting steroidogenesis and inducing apoptosis, Further, this work shows that MXC may exert toxicity via an AHR pathway.

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CHAPTER I

1.1 Introduction

The mammalian ovary contains a fixed number of different follicle types known as primordial, primary, pre-antral, and antral follicles. Follicles must grow from the primordial to the antral stage to be capable of ovulation and estrogen secretion. Thus, antral follicles play an important role in regulation of normal menstrual/estrous cyclicity, maintenance of the reproductive tract, and fertility. This follicle type is comprised of an oocyte, granulosa cells, and theca cells. The granulosa and thecal cells are responsible for production of endocrine hormones such as estradiol (E_2) (Hirshfield, 1991).

Many chemicals are known to be present in the environment. These environmental chemicals either directly affect reproductive tissues by mimicking endogenous hormones or indirectly affect reproductive tissues after conversion of the parent compound to an active metabolite, which then acts as an endocrine disrupting chemical (EDC). Animal studies have shown that in utero exposure of certain EDCs affects the development of the female reproductive system. Human beings and some wild-life species are exposed to a wide variety of chemicals through food and water. Humans may also be exposed through their occupation. Some chemicals that act as EDCs include plasticizers, pesticides, phytoestrogens, and dioxins.

The ovary is an important reproductive tissue involved in ovulation and steroidogenesis, and it contains follicles at different stages of development from primordial to antral follicles. Some EDCs target specific stages of follicle development in the ovary. The susceptibility of the follicles to harmful effects of a chemical depends on the stage of the follicle, duration of exposure, and the concentration that reaches the follicle (Sharara *et al*., 1998; Borgeest *et al*.,

2002). EDCs such as benzo(a)pyrene affect primordial and primary follicles (Mattison *et al*., 1989). Heavy metals such as lead and cadmium affect all stages of follicles (Taupeau *et al*., 2001; Junaid, 1997). 4-vinylcyclohexene (VCH) and 4-vinylcyclohexene diepoxide (VCD) affect primordial and primary follicles (Thompson *et al*., 2005; Smith *et al*., 1990; Flaws *et al*., 1994). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) affects pre-antral and antral follicles (Heimler *et al*., 1998). Organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT), kepone, lindane, and dieldrin affect many different stages of follicles (Reigart *et al.* 1999).

1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane (methoxychlor; MXC) is a pesticide that is known to target antral follicles. It is widely used in many parts of the world against insects or pests that attack crops, gardens, fruits, vegetables and animals. MXC use became widespread in the 1970s after the popular chlorinated pesticide DDT was banned in the US and other parts of the world. MXC enters the environment when it is sprayed on crops, fruits or forests. When it is aerially sprayed, MXC contaminates wide areas including bodies of water and it travels to distant areas through wind or running water. Thus, air, water or foods are the main sources of MXC exposures for human beings and wild-life species.

MXC decreases antral follicle growth, which may lead to decreased capacity to ovulate and thus, affecting fertility (Miller *et al*., 2005; Swartz and Eroschenko, 1998). MXC also targets and destroys antral follicles by causing atresia (death via apoptosis) (Borgeest *et al*., 2002). An increased rate of atresia may result in infertility as well as premature or early onset of menopause or reproductive senescence. Premature or early onset of menopause is a concern because it has been associated with an increased risk of chronic diseases such as osteoporosis and cardiovascular disease (Britt and Findlay, 2002; Couse and Korach, 1998; Findlay *et al*., 2001). While previous studies indicate that MXC slows follicle growth and destroys follicles by causing

atresia (Borgeest *et al*., 2002), the mechanism by which MXC slows follicle growth and increases atresia is unknown. Thus, the goal of this dissertation work was to understand the mechanistic pathways by which MXC causes toxicity in antral follicles. Specifically, I tested the following three main hypotheses: (1) MXC causes toxicity in antral follicles by inhibiting steroidogenesis via altering steroidogenic and metabolic enzymes, (2) MXC induces atresia of antral follicles by altering *Bcl2* members and caspase activity, and (3) MXC causes atresia and inhibits follicle growth through an aryl hydrocarbon receptor (AHR) pathway.

The present chapter details the overview of the entire dissertation. In chapter II, I have described the significance and background for the project, including the importance of the study, routes of exposure, levels of exposure, and endocrine disruptive activities caused by MXC. In chapter III, I have described the work related to the hypothesis that MXC alters steroid levels by altering steroidogenic as well as metabolic enzymes in antral follicles. In chapter IV, I have described the work related to the hypothesis that MXC induces atresia by altering expression of *Bcl2* family members and caspase activity. In chapter V, I have described the work related to the hypothesis that MXC inhibits follicle growth and induces atresia by acting through the AHR pathway. In chapter VI, I have explained the complete mechanism of action of MXC derived from our studies. In addition, I have discussed the potential pitfalls and propose future studies to complete our work so that we can better understand the mechanism of action of MXC.

To test the three main hypotheses of the dissertation, I completed the following specific aims:

Specific aim1: To determine whether MXC alters E_2 levels by regulation of synthesis and/or metabolism of E_2 in antral follicles

To complete this aim, I cultured antral follicles isolated from CD1 mouse ovaries for 24,

48, and 96 h with vehicle control (dimethylsulfoxide; DMSO) or varying concentrations of MXC (1, 10, 100 μg/ml). I then tested whether MXC decreases sex steroid levels in antral follicles and found that it reduces E_2, testosterone, and androstenedione levels. I then tested whether MXC decreases E_2 levels by altering synthetic as well as metabolic enzymes. I found that MXC decreases most of the E_2 synthetic enzymes and increases the metabolic enzyme Cyp1b1. Collectively, these data show that MXC inhibits steroidogenesis by inhibiting expression of synthetic enzymes and inducing expression of metabolic enzymes.

Specific aim 2: To determine whether MXC induces atresia by altering pro- and anti-apoptotic factors and caspase activity in antral follicles.

To complete this aim, I cultured antral follicles isolated from CD1 mouse ovaries for 24, 48, and 96 h. I first tested whether MXC induces atresia in antral follicles and found that MXC induces atresia as early as 48 h. I then tested whether MXC decreases anti-apoptotic factors (*Bcl2, Bcl2-xL*) and increases the pro-apoptotic factors (*Bax, Bok*) in antral follicles during induction of atresia. I found that MXC decreases expression of an important anti-apoptotic factor (*Bcl2*) and increases the pro-apoptotic factor (*Bax*) in follicles. In addition, I found that MXC induces caspase activities in antral follicles at all time points. Collectively, these data suggest that MXC induces atresia by increasing the ratio of *Bax/Bcl2* and inducing caspase activity.

Specific aim 3: To determine whether MXC inhibits the growth and induces atresia of antral follicles through the AHR pathway.

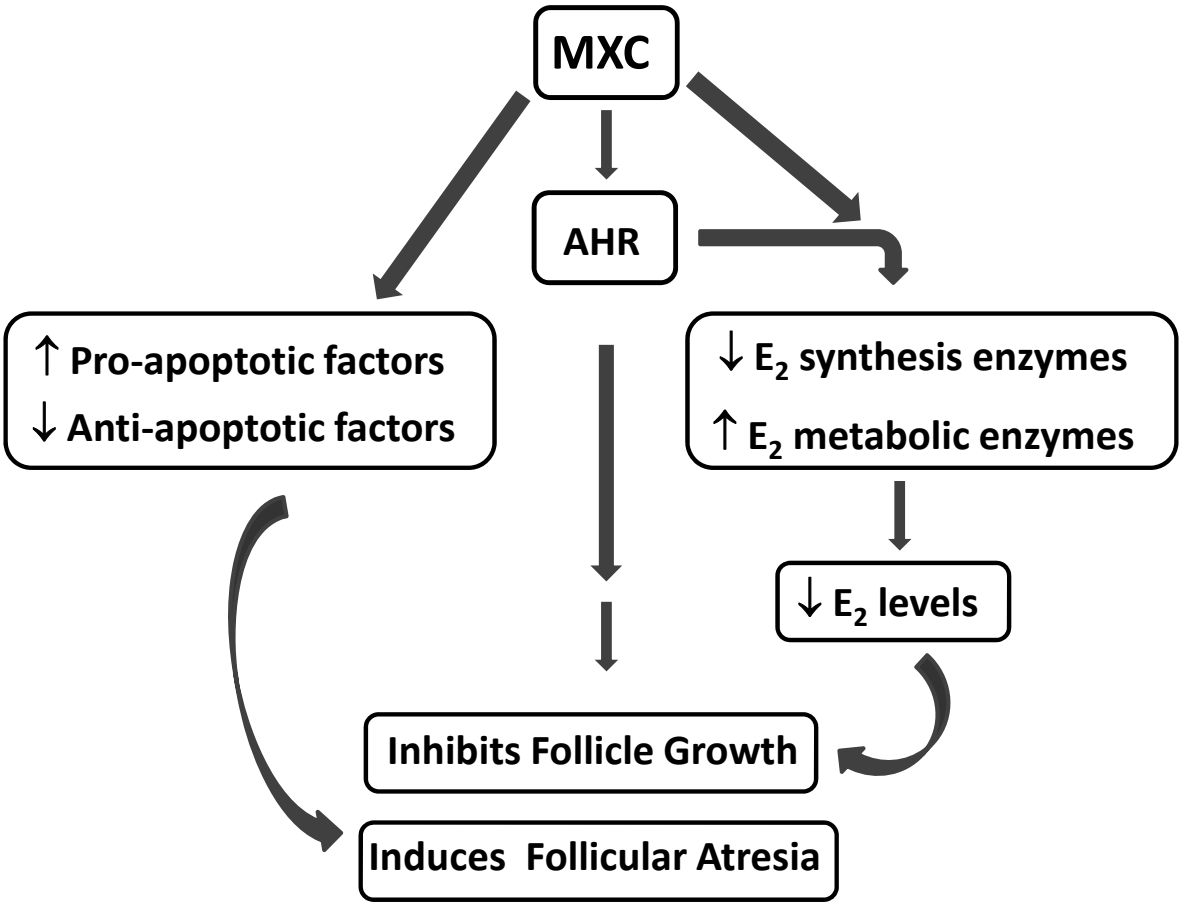
To complete this aim, I first tested whether MXC binds to AHR in ovarian cells. Briefly, I transfected granulosa cells with a xenobiotic response element (XRE) promoter containing

plasmid and found that MXC induces XREs similar to the positive control chemical TCDD. Next, I compared the ability of MXC to inhibit follicle growth and induce atresia in wild-type (WT) and AHR knockout (AHRKO) mice. I found that MXC (10 μg/ml) inhibits follicle growth and induce atresia in WT, but not in AHRKO follicles. Collectively, these results suggest that MXC inhibits follicle growth and induces atresia through an AHR pathway.

Below is the model that summarizes the main hypotheses tested in this dissertation project.

1.2 Figures

Figure 1.1 Model of action of MXC in antral follicles



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CHAPTER II

Significance and Background

2.1 Significance

MXC targets and destroys the antral follicles in the ovary (Borgeest *et al*., 2002; Swartz and Eroschenko, 1998; Swartz and Corkern, 1992). Specifically, MXC causes atresia (death via apoptosis) of antral follicles. This is of concern because antral follicles are the structural and functional units of the ovary and each mammalian ovary contains a finite number of antral follicles. The antral follicles have the ability to release eggs for fertilization and to produce sex steroid hormones. The sex steroid hormones produced by follicles are important for regulating the menstrual/estrous cycle, maintaining the reproductive tract, and initiating and maintaining pregnancy (Hirshfield, 1991). Chemicals such as MXC increase the rate at which follicles are depleted from the ovary by the process of atresia, resulting in an early menopause or early reproductive senescence. Further, atresia of antral follicles ablates fertility. The mechanism by which MXC increases atresia, however, is unknown.

The current work is significant because it tested whether the mechanism of MXC-induced toxicity involves effects of MXC on steroidogenesis and/or apoptosis and whether it involves AHR pathways. Thus, the current studies greatly improved our understanding of the mechanisms by which MXC damages the ovary, and in turn, this improved understanding may lead to the development of novel targets for the treatment and/or prevention of infertility induced by environmental chemical exposures. The present work is also significant because MXC is an excellent model compound for investigating the effects of an agent that modulates endocrine

pathways. Thus, the current studies also have increased our understanding of the mechanisms by which endocrine disruption adversely affects ovarian function. In addition, our studies with MXC lead to further insights into the ligand specificity for AHR; thereby increasing our understanding of the physiological and toxicological roles of the receptor. This improved understanding may lead to the development of novel ligands for the AHR, which may be used to treat infertility or may be used as contraceptive agents. The present work also showed that MXC induces atresia in antral follicles by altering Bcl2 pathways. These results are important as interruption of these apoptotic pathways could rescue follicles from MXC-induced atresia and thus improve the fertility in the exposed groups. However, future studies are required to develop methods to minimize or prevent the toxicity of MXC on fertility.

2.2 Background

Ovarian Physiology and Atresia

The ovaries of female mammals contain a fixed number of primordial follicles at the time of birth (Hirshfield, 1991; Greenfeld and Flaws, 2004). These primordial follicles grow into more mature follicle stages known as primary, pre-antral, and antral follicles (Figure 2.2). These follicles contain the female germ cell (oocyte) with the somatic cells surrounding it. Follicles are classified based on the type and number of layers of somatic cells. The earliest stage of follicle is primordial follicle, which contains an oocyte surrounded by a single layer of fusiform granulosa cells. Some primordial follicles will grow into the primary stage, which is defined by a slightly larger oocyte surrounded by a single layer of cuboidal granulosa cells. After the primary stage, follicles grow into the pre-antral stage, which is characterized by an oocyte surrounded by 2-4 layers of granulosa cells and a theca cell layer. Pre-antral follicles then grown into antral

follicles, which contain an oocyte surrounded by several layers of granulosa cells, a fluid filled antral space antrum, and two distinct layers of thecal cells. Antral follicles are the only type of follicles capable of releasing fertilizable oocyte and they are the major site of synthesis of steroid hormones such as estrogens. The estrogens produced by antral follicles are essential for normal menstrual and estrous cyclicity, maintenance of the female reproductive tract, and maintenance of non-reproductive tissues such as bones, vascular tissues, and the brain (Hirshfield, 1991; Britt and Findlay, 2002; Findlay *et al*., 2001; Couse and Korach, 1998).

During the reproductive lifespan, a constant stream of follicles grows from the primordial to the antral stage. Although a few antral follicles ovulate and become fertilized, approximately 99% of all follicles die via a process known as atresia. Many chemicals are known to affect follicular growth and atresia; thus, affecting reproductive function. Chemicals such as MXC are of concern because they increase the rate of atresia of follicles. In turn, this leads to acceleration in the rate of depletion of follicles, resulting in an early menopause or early reproductive senescence. Early menopause is a concern because it has been associated with an increased risk of chronic diseases such as osteoporosis and cardiovascular disease (Sowers and La Pietra, 1995).

MXC

Biochemistry of Methoxychlor

The structure of MXC is shown in figure 2.1. The oral lethal dose$_{50}$ (LD_{50}) of MXC in rat is >6000 mg/kg, making this chemical less acutely toxic than DDT, which has an oral LD_{50} of 118 mg/kg (ATSDR, 2002). Animal studies suggest that MXC is converted to more soluble metabolites in the liver. MXC and its metabolites are excreted mainly through feces and to a

lesser extent in urine within 24 h of ingestion (Kapoor *et al*., 1970). In livers of rats and humans, 1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane (methoxychlor; MXC) converts to mono- and the bis-demethylated metabolites: 1,1,1-trichloro-2(4-methoxyphenyl),2(4-hydroxyphenyl)ethane (mono-OH-MXC) and 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane (HPTE) (Stresser and Kupfer, 1998; Kapoor *et al*., 1970). CYP1A2 and CYP2C19 are involved in demethylation of MXC, forming mono- and bis-demethylated metabolites in tissues (Stresser and Kupfer, 1998). Further, the mono- and bis-demethylated metabolites are metabolized to the more polar ring hydroxylated metabolite tris-OH-MXC by CYP3A4 enzymes (Stresser and Kupfer, 1997, 1998; Kupfer *et al*., 1990).

I focused my project on MXC for several reasons. First, MXC is widely used in many countries against insects that attack fruits, vegetables, and home gardens. MXC has been found in 1.2% composite food samples in the US (Badach *et al*., 2000, Badach *et al.* 2007). One study in Poland showed that the MXC concentration in water reached up to 0.31 μg/l (Buczynska and Szadkowska-Stannczyk, 2005). A study in Connecticut showed that up to 35% of agricultural commodities contain pesticide residues and that MXC is present in lettuce, strawberries, spinach, and peas (Krol *et al*., 2000). When it is aerially sprayed, MXC contaminates wide areas including water bodies and it travels to distant areas through wind or running water. Thus, air, water or foods are the main sources of MXC exposures for human beings and other species. Due to failure of manufacturer registration with the Environmental Protection Agency (EPA), MXC production has been stopped in the US (Mulkey, 2002). However, MXC is still used in many countries for agricultural products that are imported in to the US, resulting in human exposure in the US. Further, MXC persists in soil, and residues are present even after 18 months of post treatment of microbes that scavenge MXC (Golovleva *et al*., 1984).

Second, humans are exposed to MXC through food and water consumption (Campoy *et al*., 2001). The half life of MXC is 24 h and most of its metabolites leave the body primarily through feces and secondarily through urine. The EPA limits the amount of MXC present in drinking water to be 0.04 parts of MXC per million parts of water (ppm) and it has also set the limits in agricultural products (crops, fruits, vegetables, grains, meats, milk, and food for livestock) to be 1-100 ppm (ATSDR, 2002). An FDA survey has indicated that the average intake of MXC in foods is 0.8 μg/day or about 292 μg/year. People working in manufacturing industries and who live near farms have great chances of high exposure to MXC. Occupational exposure to MXC in farm people has been measured to be 5.16 μg/ml in serum (ATSDR, 2002). Once MXC enters the body, it reaches serum, placenta, breast milk, and adipose tissue. One study has shown that the levels of MXC can reach 0.39 ng/ml in serum and 155.58 μg/kg in adipose tissue (Botella *et al*., 2004). Many studies have shown that the concentration of MXC can reach 10.41μg/kg in placenta (Lopez-Espinosa *et al*., 2007), 1.01 ng/ml in breast milk, 2.49 ng/ml in transition milk, and 18.1 ng/ml in colostrum (Campoy *et al*., 2001). Further, one study has detected several pesticide residues including MXC in milk and it also found an association between breast milk exposure of organochlorine pesticides and cryptorchidism (Damgaard *et al*., 2006).

Third, I selected MXC for the study because it is known to be a female reproductive toxicant (Eroschenko and Cooke, 1990; Symonds *et al*., 2006; Borgeest *et al*., 2004). Several studies have reported that MXC reduces fertility in variety of species (Gupta *et al*., 2007; Cummings and Gray, 1989). Studies have shown that MXC is estrogenic in the uterus and anti-estrogenic in the ovary. In utero exposure to MXC increased atretic follicles in F1a litters and the residual effect of MXC induced advanced vaginal opening in F1b litters. In addition, mothers

exposed to MXC have an increased gestation period and increased number of dead fetuses compared to vehicle controls during the first pregnancy (Swartz and Corkern, 1992). In sheep, prenatal exposure to MXC delays the luteinizing hormone surge in offspring, suggesting that MXC exposure has long-term endocrine effects that control fertility (Savabieasfahani *et al*., 2006). Neonatal exposure to MXC in mice caused no problems with mating, but decreased ovulation and pregnancy rate. In addition, MXC also decreased the number of live fetuses and increased resorbed fetuses (Swartz and Eroschenko, 1998). In another study, neonatal exposure to high doses of MXC (67.6 and 135.1 mg/kg/d) in female mice also induced ovarian atrophy, decreased relative ovarian weight, and decreased corpora lutea (CL) numbers, whereas exposure to low doses of MXC (6.8 and 13.5 mg/kg/d) showed opposite effects, and the ovaries contained large, heavy, and increased CL numbers (Eroschenko *et al*., 1995, Eroschenko *et al*., 1997). Further, MXC exposure on day 1 through 4 of pregnancy inhibits implantation in mice. In addition, it also affects uterine receptivity and embryonic development and transport, suggesting estrogenic action of MXC at the level of the uterus and oviduct (Hall *et al*., 1997). In adult female mice, MXC induces persistent vaginal estrus, decreases ovarian weight, and increases the number of atretic large follicles compared to vehicle controls (Martinez and Swartz, 1992). Specifically, MXC has been shown to target antral follicles, reducing the number of healthy antral follicles and increasing the atresia of follicles. MXC-induced atresia of antral follicles involves Bcl2 and oxidative stress pathways and it has been shown to inhibit antral follicle growth in vitro (Borgeest *et al*., 2002, Borgeest *et al*., 2004; Gupta *et al*., 2006; Miller *et al*., 2005).

Previous studies have demonstrated various effects of MXC on male reproductive functions. Exposure to MXC in male rats from weaning through adulthood exhibited delayed

puberty, decreased epididymal weight, and decreased number of sperm in the ejaculate, without change in sperm number in testis (Gray *et al*., 1989). Fetal exposure to low doses of MXC increased the prostate size in adult mice (Welshons *et al*., 1999). Also, exposure to MXC reduced seminal vesicle weight, serum testosterone, dehydroepiandrosterone levels and increased follicle-stimulating hormone levels (Murono *et al*., 2006). Perinatal and juvenile exposure to MXC reduced testicular weight, number of epididymal spermatozoa, and sertoli cell numbers (Larry *et al*., 2002).

MXC has been shown to alter oxidative stress and cell cycle regulators in the ovary. MXC increases H_2O_2, reactive oxygen species (ROS), and also decreases mRNA expression and activity of antioxidants such as Cu/Zn superoxide dismutase (SOD1), glutathione peroxidase (GPX), and catalase (CAT) in the mouse ovary. Collectively, these studies suggest that MXC induces apoptosis in the ovary by inducing the oxidative stress disrupting the mitochondrial homeostasis (Gupta *et al*., 2006a). Exposure to MXC in vivo and in vitro reduces expression of cell cycle regulators: PCNA protein and *Ccnd2* and *Cdk4* in ovarian tissues (Gupta *et al*., 2009). In addition, treatment with the anti-oxidant N-acetyl cysteine increases *Ccnd2* and *Cdk4* levels in MXC-treated antral follicles, suggesting that MXC-induced oxidative stress decreases the levels of cell cycle regulators, which in turn, results in inhibition of the growth of antral follicles (Gupta *et al*., 2009; Gupta *et al*., 2006b).

Fourth, I elected to study MXC because it is considered to be a model endocrine disruptor and environmental estrogen (Cummings, 1997). MXC is considered to have estrogenic as well as anti-estrogenic activities depending on the species and tissue type (Ostby *et al*., 1999; Gray *et al*., 1988; Hall *et al*., 1997). These endocrine disrupting activities have been suspected to be the reason that MXC causes infertility. Thus, studying the model endocrine disruptor MXC will be

useful for identifying the mechanisms by which environmental estrogenic and anti-estrogenic compounds affect ovarian function and/or fertility.

A final reason for focusing on MXC is that many wild-life species have been exposed to MXC and are at great risk of reproductive damage. One study has found MXC and other organochlorine residues in finfish in Maryland waters (Eisenberg and Topping, 1985). Another study on Michigan Rivers has found MXC in fish (Giesy *et al*., 1994). Further, many studies have found MXC in crocodiles from Costa Rica and Belize, in elephant seals in Antarctica, and in Arctic beluga whales (Rainwater *et al*., 2007; Wu *et al*., 2006; Miranda-Filho *et al*., 2007; Stern *et al*., 2005). Thus, by understanding the mechanism of MXC action, it may be possible to determine the potential impacts of environmental chemicals on wide variety of species.

MXC and Estrogen levels

Estrogens are important in maintaining physiology of a vast number of tissues, including reproductive tissues. Estrogens are involved in the maintenance of the estrous cycle, growth of follicles, ovulation, fertility, and maintenance of pregnancy. Estradiol (E_2) is the principal estrogen formed in the body and is synthesized from cholesterol. In theca cells, cholesterol is transported from the outer region to inner portion of mitochondria by steroid acute regulatory protein (StAR) protein and is converted to pregnenolone by CYP450 side chain cleavage enzyme ($CYP450_{scc}$). Then two enzymatic pathways convert pregnenolone to androstendione. In one pathway, 3β-hydroxysteroid dehydrogenase (3β-HSD) catalyzes the conversion of pregnenolone to progesterone. The progesterone is then converted to androstenedione via 17α-hydroxylation of progesterone by 17α hydroxylase and 17, 20 lyase (CYP17α). In the second pathway, pregnenolone is converted to 17α-hydroxypregnenolone and then to dihydroepiandrosterone

(DHEA) by CYP17α. The DHEA is then finally converted to androstenedione by 3βHSD. Androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase. Further, testosterone is transferred to granulosa cells where it is converted to estrogens by aromatase enzyme (CYP19). The estrogens are then released into the blood stream to carry out a variety of functions in the body (Suter, 2004) (Figure 2.3).

Many previous studies have shown that several environmental toxicants are involved in alteration of E_2 levels. Previous studies have shown that bisphenol A decreases E_2 levels including other intermediates of the E_2 steroidogenic pathway in the ovarian antral follicles (Peretz *et al*., 2011). Studies have shown that di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate reduce E_2 levels in antral follicles and granulosa cells of the ovary (Gupta *et al*., 2010; Lovekamp and Davis, 2001). Exposure to cigarette smoke also has been associated with decreased E_2, estrone and estriol levels in humans (Soldin *et al*., 2011). Importantly, studies have shown that exposure to MXC and its metabolites causes reduction in E_2 levels. Studies in rat has shown that MXC does not alter E_2 levels in serum, but decreases E_2 levels in ovarian culture (Cummings and Laskey, 1993) and studies on bovine granulosa cells also have shown that MXC (1 μg/ml) decreases E_2 levels (Tiemann *et al*., 1996). Mono-hydroxy MXC a metabolite of MXC has also been shown to decrease E_2 levels in antral follicles of the ovary (Craig *et al*., 2010).

Our data indicated that MXC reduces E_2 levels in antral follicles in vitro. Based on such data, my hypothesis was that MXC reduces estradiol levels either by alteration of CYP 450 enzymes involved in E_2 biosynthesis pathway or up-regulation of CYP450s involved in E_2 metabolism. Thus, the present work examined both E_2 biosynthesis and metabolism.

MXC and the Apoptotic Pathway

Apoptosis or programmed cell death is a normal physiological process that occurs in all multicellular organisms to remove cells that are superfluous, have already completed their specific functions, or are harmful to the whole organism (Thompson, 1995; Kerr *et al*., 1972). During reproductive life, over 99% of ovarian follicles undergo atresia at the early antral follicle stage (Hsu and Hsueh, 1998; Hsueh *et al*., 1996). The process of atresia occurs via apoptosis. Apoptosis of ovarian follicle cells is mainly regulated by pro-survival hormones such as gonadotropins, which act on the ovary to produce paracrine and autocrine factors such as estrogens, cytokines, interleukin-1, nitric oxide, and insulin-like growth factor I. It is likely that none of these factors is singularly obligatory in the control of follicle growth or demise. Instead, a balance of these different survival and apoptotic factors may decide whether a follicle will continue development or undergo apoptosis.

Several anti-apoptotic factors Bcl2, Bcl-xL, and Mcl-1 help in cell survival, whereas pro-apoptotic factors such as Bax, Bak, and Bok antagonize cell survival and induce apoptosis (Hsu *et al*., 1997; Chittenden *et al*., 1995; Oltvai *et al*., 1993). In a biological system, it is the ratio of Bcl2:Bax that determines the fate of the cell either to death or survival. When Bcl2 is in excess, either Bcl2:Bax heterodimers or Bcl2 homodimers predominate and the cells are protected. When Bax is in excess, Bcl2:Bax heterodimers or Bax homodimers predominate and the cells are prone for apoptosis (Basu and Haldar, 1998). In Bcl2 overexpressors, there is a suppression of follicular cell apoptosis and enhancement of folliculogenesis, suggesting involvement of the Bcl2 system in the ovary (Hsu *et al*., 1996). In addition, in mice with elevated Bcl2, there is a suppression of apoptosis, which is induced by external agents both in vivo and in vitro (Baffy *et al*., 1993; Allsopp *et al*., 1993; Nunez *et al*., 1991). Mice deficient in Bcl2 do not show any

abnormality during development, but only few tissues including hair follicles, lymphoid systems, and kidney show apoptosis (Veis *et al*., 1993; Sorenson *et al*., 1995). In addition to Bcl2, several other proteins such as Bcl-xL, Mcl-1, and Bfl-1 have similar anti-apoptotic activity by interacting with Apaf-1, preventing activation of caspases, thus, suppressing the caspase cascade and apoptosis (Hsu and Hsueh, 2000). Expression of Bcl-xL can lead to significant resistance to apoptosis at least as much as Bcl2 (Boise *et al*., 1993). The importance of Bax was explained by increase in number of primordial follicles in Bax deleted mice (Perez *et al*., 1999). Another pro-apoptotic factor Bok preferentially dimerizes with Mcl-1, Bfl-1, but not with Bcl-2, Bcl-w, and Bcl-xL (Inohara *et al*., 1998). Survival factors such as gonadotropins increase the phosphorylation of BAD; thus, dampening its killing ability (Del Peso *et al*., 1997; Zha *et al*., 1996). The pro-apoptotic factors such as Bax, Bak, and Bok dimerize with anti-apoptotic proteins, relieving the Bcl2 suppressed Apaf-1 and altering the membrane homeostasis, thus increasing cytochrome *c* release inducing the caspase cascade and apoptosis. The released cytochrome *c* binds to Apaf-1 and procaspase-9 to form active apoptosome complex. Activated caspase-9, in turn cleaves and activates caspase-3 and other caspases. These activated caspases in turn increase the mitochondrial permeability by converting the anti-apoptotic factors into pro-apoptotic factors and ligands. Finally, activated caspases cleave several proteins and DNA leading to formation of apoptotic bodies (Hsu and Hsueh, 2000).

In addition to being important regulators of natural atresia, Bax and Bcl2 are involved in chemical induction of apoptosis. The industrial chemical, 4-vinylcyclohexene diepoxide (VCD) has been shown to induce atresia in small pre-antral follicles by altering Bcl2 family members (Hu *et al*., 2001). The polyaromatic hydrocarbon dimethylbenz(*a*)anthracene also has been shown to increase Bax levels, inducing apoptosis in mouse oocytes (Matikainen *et al*., 2001).

Further, chromium has been shown to increase Bax and decrease Bcl2, inducing apoptosis in mouse ovarian granulosa cells (Banu *et al*., 2011). Importantly, studies involving in vivo and in vitro MXC exposure showed that apoptosis was increased, with an increase of Bax and with no alteration or decrease in Bcl2 levels (Miller *et al*., 2005; Borgeest *et al*., 2002).

The importance of pro-apoptotic and anti-apoptotic factors in chemical induction atresia, made us to examine these factors in response to MXC. Thus, our study examined and found that MXC alters *Bcl2* and *Bax* factors and increase caspase activities, which further induce atresia in antral follicles.

Methoxychlor and the Aryl Hydrocarbon Receptor Pathway

These studies focused on whether MXC mediates toxicity via AHR. The AHR is a ligand activated transcription factor present in the cytoplasm of cells, which exists as a complex with heat shock protein 90, immunophilin like protein XAP2, and co-chaperone p23. Once ligand binds to the AHR complex, it translocates into the nucleus, where the ligand-AHR is released from the complex and heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT). The newly formed ligand-AHR-ARNT complex then binds to AHR response elements adjacent to target promoters of various genes and initiates transcription. There are numerous studies showing that activation of the AHR complex initiates transcription of various cytochrome P450 family of genes such as Cyp1a1, Cyp1a2, and Cyp1b1, which are involved in metabolism of various xenobiotics (Hernandez-Ochoa *et al*., 2009; Whitlock, 1999; Safe, 1995).

The present study determined whether MXC works through the AHR pathway. The AHR pathway was selected for several reasons. First, many studies have demonstrated cross-talk between AHR-ER pathways in many tissues (Buchanan *et al*., 2000; Safe *et al*., 1998; Wormke

et al., 2000). The cross talk between AHR-ER pathways may alter the ability of MXC to reduce estradiol levels, induce inhibitory factors, and/or degrade receptors during the process of antral follicle toxicity.

Second, many environmental toxicants belonging to a group of polyhalogenated aromatic hydrocarbons (PAHs) such as the polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins, and polychlorinated dibenzofurans that have been shown to bind to the AHR (Schmidt and Bradfield, 1996). Poland and co-workers were the first to show that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) binds to AHR in hepatic cytosols (Poland *et al*., 1976). Many other studies have shown that PAHs such as benzo(*a*)pyrene, 7,12-dimethybenz(*a*)nthracene, and 3-methyl cholanthrene decrease the number of primordial and primary follicles at birth. Their effects were reduced by treatment with the AHR antagonist α-napthoflavone (ANF), thus, suggesting that these PAHs bind to AHR (Shiromizu and Mattison, 1984, Shiromizu and Mattison, 1985). Studies have also shown that AHR disruption by ANF reduces the ability of 9,10-dimethylbenz(*a*)antracene-3,4-dihydrodiol (DMBA-DHD) to cause cell death and as a result increases the number of fetal oocytes (Matikainen *et al*., 2002; Matikainen *et al*., 2001). Studies in granulosa cells also have shown that TCDD induces *Cyp1a1* and *Cyp1b1* by increasing expression of *Ahr* (Dasmahapatra *et al*., 2001). Binding of toxicants to the AHR induces metabolic enzymes, leading to a variety of toxic effects. MXC is similar in structure to many known AHR ligands. Thus, it is possible that MXC may bind to the AHR.

Third, one study suggests that the AHR mediates the toxicity of MXC in liver cells (Han *et al*., 2007). Han et al. have shown that TCDD induces CYP1A1 expression in mouse liver cells. TCDD is the classical ligand known to bind to the AHR and then to induce CYP1A1 expression. MXC alone did not block CYP1A1 expression, but when used with TCDD, MXC inhibited the

expression of CYP1A1 by blocking the binding potency of the AHR to the dioxin response element (DRE) present upstream of CYP1A1 gene. Thus, this raises the possibility of action of MXC through the AHR. Our study shows that MXC binds to AHR, inhibiting follicle growth and inducing atresia in mouse ovarian antral follicles.

2.3 Figures and Legends

Figure 2.1 Structure of MXC

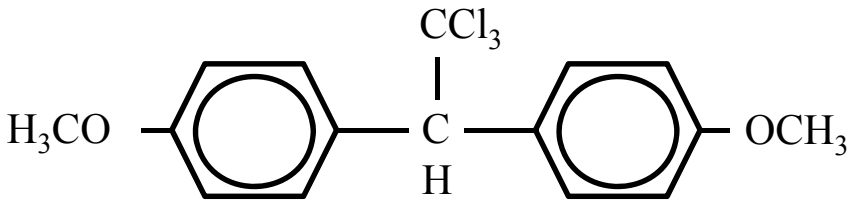
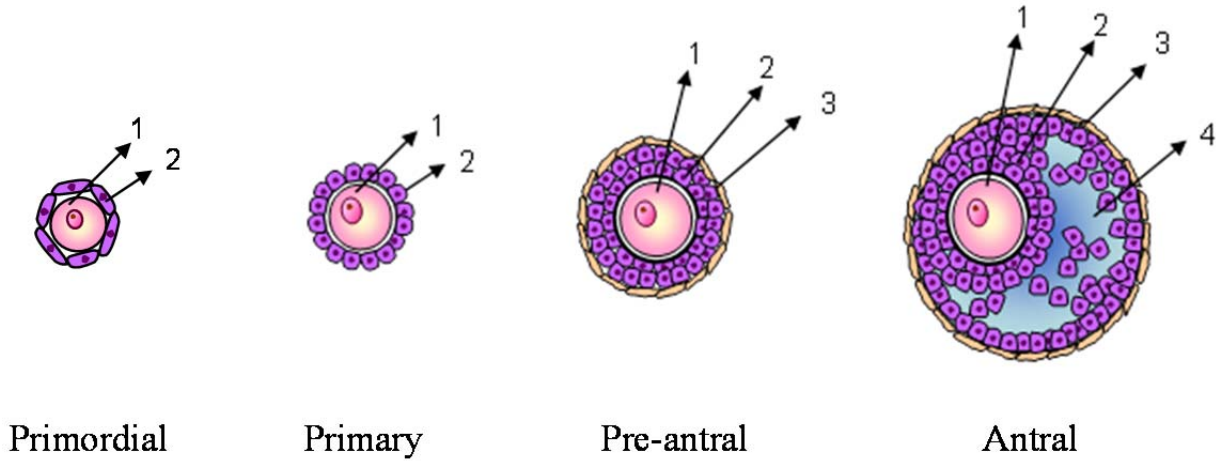


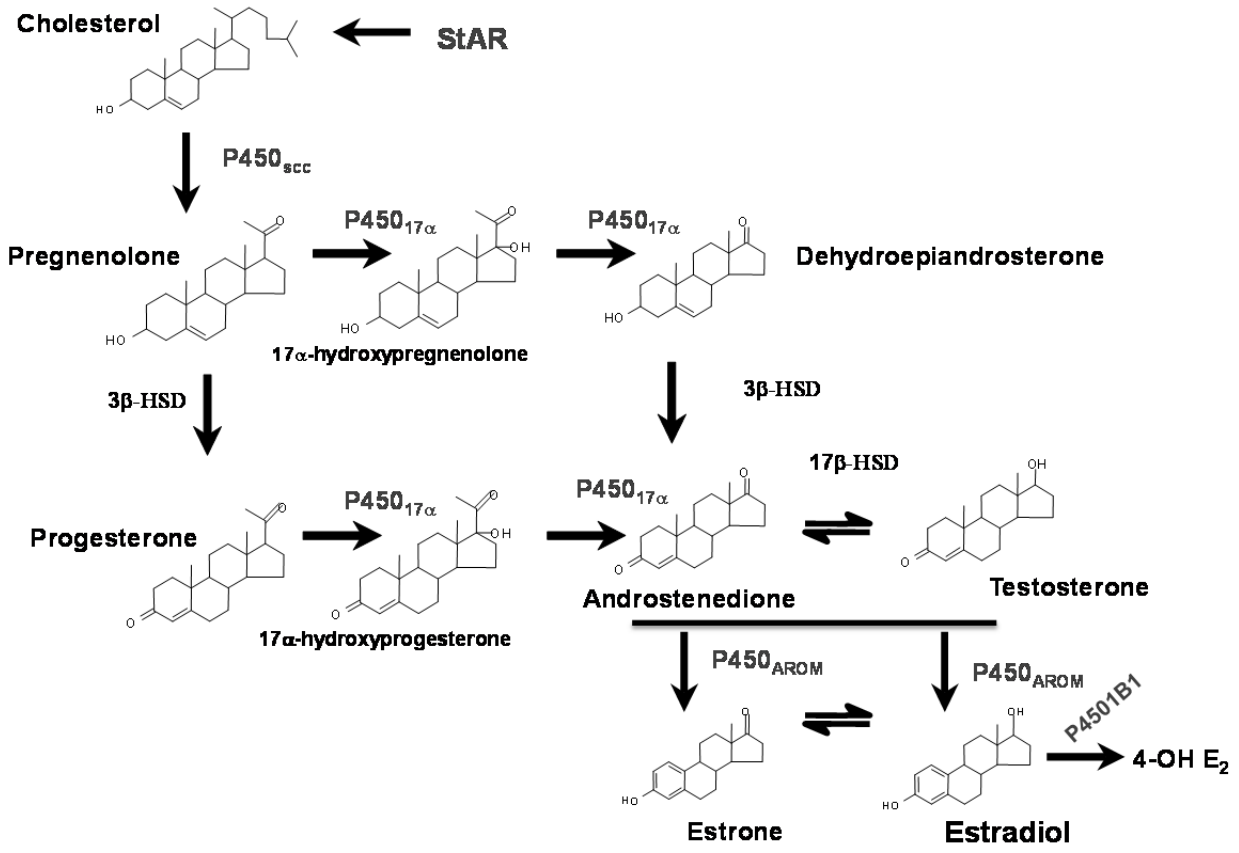
Figure 2.2 Stages of Follicular Development in the Mammalian Ovary.



1=Oocyte, 2= Granulosa cell layer, 3= Theca cell layer, 4= Antrum

The ovary is composed of different stages of follicles. The earliest stage of the follicles is the primordial follicle followed by the primary and pre-antral follicle and finally, the antral follicle.

Figure 2.3 Schematic of the Estradiol Bio-synthesis Pathway in the Ovary



In thecal cells, cholesterol is converted to androgens: androstenedione and testosterone; Then these androgens transported to granulosa cells, where they are converted to estrone and estradiol. Both thecal cells and granulosa cells are integral part of isolated follicles used in our experiments.

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CHAPTER III

Methoxychlor reduces estradiol levels by altering steroidogenesis and metabolism in mouse antral follicles in vitro

3.1 Abstract

The organochlorine pesticide methoxychlor (MXC) is a known endocrine disruptor that affects adult rodent females by causing reduced fertility, persistent estrus, and ovarian atrophy. Since MXC is also known to target antral follicles, the major producer of sex steroids in the ovary, the present study was designed to test the hypothesis that MXC decreases estradiol (E_2) levels by altering steroidogenic and metabolic enzymes in the antral follicles. To test this hypothesis, antral follicles were isolated from CD-1 mouse ovaries and cultured with either vehicle (dimethylsulfoxide; DMSO) or MXC. Follicle growth was measured every 24 h for 96 h. In addition, sex steroid hormone levels were measured using enzyme-linked immunosorbent assays (ELISA) and mRNA expression levels of steroidogenic enzymes as well as the E_2 metabolic enzyme *Cyp1b1* were measured using qPCR. The results indicate that MXC decreased E_2, testosterone, androstenedione, and progesterone (P_4) levels compared to DMSO. In addition, MXC decreased expression of aromatase (*Cyp19a1),* 17β-hydroxysteroid dehydrogenase 1 (*Hsd17b1),* 17α-hydroxylase/17,20-lyase (*Cyp17a1*), 3β hydroxysteroid dehydrogenase 1 (*Hsd3b1),* cholesterol side-chain cleavage (*Cyp11a1),* steroid acute regulatory protein (*Star)*, and increased expression of *Cyp1b1* enzyme levels. Thus, these data suggest that MXC decreases steroidogenic enzyme levels, increases metabolic enzyme expression and this in turn leads to decreased sex steroid hormone levels.

3.2 Introduction

Many chemicals are known to be present in the environment. Many of them mimic endocrine hormone functions in the body, interfering with normal endocrine activity in humans, wild-life, or laboratory animals, thus, acting as endocrine disrupting chemicals (EDCs) (Crisp *et al*., 1998). The EDC methoxychlor (MXC) is a chlorinated organic pesticide, which is primarily used against various species of insects that attack field crops, trees, vegetables, fruits, gardens, stored grain, livestock and domestic pets (ATSDR, 2002). This chemical was first registered for use in 1948 and was widely used as a replacement for dichlorodiphenyltrichloroethane (DDT) until 2004 (Stuchal *et al*., 2006). MXC is still being used in many countries on agricultural products that are imported to the United States (US), resulting in human exposure in the US. Further, MXC is persistent in soil and its residues are present even after 18 months of post-treatment with microbes that scavenge MXC (Golovleva *et al*., 1984).

MXC targets the ovary and its exposure has adverse effects on reproductive function in adult female mice causing persistent estrus, reduced fertility, ovarian atrophy, and increased follicular atresia (Eroschenko *et al*., 1997; Martinez and Swartz, 1991). Exposure of pregnant mice to MXC results in an increased percentage of atretic follicles in female pups compared to controls. Further, the next generation of female pups born to the same mother display residual effects of MXC, showing accelerated vaginal opening (Swartz and Corkern, 1992). Exposure of adult mice to MXC increases lipid accumulation in the interstitial tissue and thecal cells of the ovary, mimicking estrogen effects (Martinez and Swartz, 1992). Further, MXC increases the number of pyknotic bodies in mouse granulosa cells, decreases the number of healthy antral follicles, increases the number of atretic follicles, and increases ovarian surface epithelium height (Borgeest *et al*., 2002; Swartz and Corkern, 1992). Under in vitro conditions, treatment of antral

follicles with MXC decreases antral follicle growth and increases atresia (Gupta *et al*., 2006; Miller *et al*., 2005, 2006).

The effects of MXC on antral follicles are of concern because antral follicles are the structural and functional units of the ovary. Further, antral follicles are the only follicle types that have the ability to release eggs for fertilization and to produce sex steroid hormones such as 17β-estradiol (E_2). The E_2 produced by antral follicles is essential for normal menstrual and estrous cyclicity, maintenance of the female reproductive tract, and maintenance of non-reproductive tissues such as bones, vascular tissues, and the brain (Britt and Findlay, 2002; Couse & Korach, 1998; Findlay *et al*., 2001).

E_2 production by antral follicles is a complex process in which cholesterol is eventually converted to E_2. During steroidogenesis, the thecal cells in the follicle acquire cholesterol through endocytosis from blood and this cholesterol is stored in the cytoplasm as cholesterol esters. Cholesterol has to be transported across both mitochondrial membranes, and this process is aided by steroidogenic acute regulatory protein (STAR). The cholesterol side-chain cleavage enzyme (CYP11A1) is a rate limiting enzyme present in all the steroidogenic cells. This enzyme is attached to the inner mitochondrial membrane and converts cholesterol to pregnenolone. The next enzyme in the steroidogenesis pathway is 3β-hydroxysteroid dehydrogenase (HSD3B1), which catalyzes the conversion of pregnenolone to progesterone (P_4). Progestins, pregnenolone and P_4 are converted irreversibly to androstenedione by 17α-hydroxylase/17,20-lyase (CYP17A1). Androstenedione is then converted to the more potent androgen testosterone by 17β-hydroxysteroid dehydrogenase (HSD17B1). This enzyme also catalyzes the conversion of estrone to a more potent estrogen, E_2. The androgens are then converted to estrogens by

aromatase (CYP19A1) (Suter, 2004). E_2 then can be metabolized to catecholestrogens (Spink *et al*., 1994).

Some studies have shown that MXC alters steroidogenesis in isolated granulosa cells and whole ovaries, thus acting as an endocrine disruptor (Chedrese and Feyles, 2001; Cummings, 1997). However, little is known about the effects of MXC on steroid production by antral follicles in mice. Therefore, the present study tested the hypothesis that MXC decreases the sex steroid hormone levels (E_2, testosterone, androstenedione, and P_4) produced by antral follicles in vitro. The present study also evaluated the mechanism by which MXC alters steroidogenesis by examining the effects of MXC on steroidogenic and metabolic enzymes.

3.3 Materials and Methods

Chemicals: MXC (99% pure) was purchased from Chemservice (West Chester, PA). Stock solutions of MXC for in vitro experiments were prepared using dimethylsulfoxide (DMSO) (Sigma, St. Louis MO) as a solvent, and in various concentrations (2, 20, and 200 mg/ml) that permitted an equal volume of solvent to be added to individual culture wells for each treatment group. Thus, final concentrations of MXC in culture were 1, 10, and 100 μg/ml (ppm). The doses used in these experiments were selected based on previously published studies showing that these concentrations of MXC induce toxicity in antral follicles, granulosa cell culture models, and in uterine leiomyoma cells (Chedrese and Feyles, 2001; Gupta *et al*., 2006; Miller *et al*., 2005). These concentrations are relevant to occupational exposure levels. The FDA monitored the chemical contaminants in food products in the United States and calculated the average daily intake of methoxychlor in adults was up to 4 ng/kg/day (ATSDR, 2002). Normally, serum levels were found to be below the level of detection. However, a study involving an occupational

exposure in farm workers has shown that MXC concentration in serum can reach up to 5.16 µg/mL (ATSDR, 2002). Thus, the occupational exposure dose is much higher than normal human exposure and lies between the doses used in the present experiments: MXC 1 µg/ml and MXC 10 µg/ml. For controls and MXC treatment groups, DMSO was used at 0.05%, which is able to solubilize MXC in aqueous media. DMSO, ITS (insulin, transferrin, selenium), penicillin, and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). Alpha-minimal essential media (α-MEM) was obtained from Invitrogen (Carlsbad, CA). Human recombinant follicle stimulating hormone (rFSH) was obtained from Dr. A.F. Parlow from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). Fetal bovine serum (5% FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

Animals: Adult female cycling CD-1 mice were purchased from Charles River Laboratories (Charles River, CA) and housed in the core animal facility located at College of Veterinary Medicine, University of Illinois and maintained on 12L:12D cycles. Mice were housed in the animal facility for at least two days to relieve transportation stress, given *ad libitum* food and water, and temperature was maintained at 22±1 °C. Animals were euthanized at 35-39 days of age by carbon dioxide (CO_2) inhalation followed by cervical dislocation. The ovaries were removed and antral follicles were isolated as explained below. The University of Illinois Institutional Animal Care and Use Committee approved all protocols involving animal care, euthanasia, and tissue collection.

Antral follicle culture: Ovaries were removed and antral follicles were isolated from ovaries of mice between 35-39 days old because this time point was used in previous studies, and this is the

age at which mice are cycling regularly (Borgeest *et al*., 2004; Miller *et al*., 2005). Antral follicles were isolated mechanically from the ovaries based on relative size and interstitial tissue was removed using fine watch maker forceps. About 3-4 mice were used per experiment and they yielded approximately 20-30 follicles per mouse. Once follicles were isolated, they were placed individually in wells of a 96-well culture plate with 150 µl of unsupplemented α-MEM prior to treatment. Each treatment group in an experiment consisted of 10-15 follicles. Supplemented α-MEM was prepared with: 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium), 100 U/ml penicillin, 100 mg/ml streptomycin, 5 IU/ml rFSH and 5% FBS. A dose response regimen of MXC (1-100 µg/ml) and vehicle controls (DMSO) was individually prepared in supplemented α-MEM. For treatment, unsupplemented α-MEM was removed from each well and replaced with 150 µl of supplemented α-MEM containing MXC or DMSO. Follicles were then incubated for 24, 48, and 96 hours (h) at 37°C in 95% air and 5% CO_2. Non-treated controls (supplemented media alone) were used in each experiment as a control for culture conditions. At the end of 24, 48, and 96 h follicle cultures, media were collected and stored at -80^{o}C for later use. In addition, follicles were collected, snap frozen, and stored at -80^{o}C for later use.

Analysis of follicle growth: Antral follicles were cultured as described above for 96 h. Follicle growth was examined in 24 h intervals by measuring follicle diameters across perpendicular axes with an inverted microscope equipped with a calibrated ocular micrometer. Antral follicles were considered as those follicles having diameters of 200 µm or greater (Miller *et al*., 2006), which correlates with the histological appearance of antral follicles. At least three separate culture experiments were performed for each chemical treatment. In the present study,

we first averaged the sizes of follicles in each treatment group within each experiment and then data were averaged and analyzed across different experiments (n=3 separate experiments).

Hormone measurements: The media samples from at least 9-12 individual wells distributed equally across 3-4 experiments were randomly selected and subjected to enzyme-linked immunosorbent assays (ELISA) as described previously (Craig *et al*., 2010). E_2, testosterone, androstenedione, and P_4 levels were measured in the media using kits from DRG International (Mountainside, NJ). These sex steroid hormones were selected since they are the major intermediates in the biosynthesis of E_2 (Jones and DeCherney, 2005). The sensitivities for each kit were 9.714 pg/ml for E_2, 0.083 ng/ml for testosterone, 0.019 ng/ml for androstenedione, and 0.045 ng/ml for P_4. The intra-assay coefficients of variation (CVs) were 4.7% for E_2, 7.1% for testosterone, 10.2% for androstenedione, and 11.4% for P_4. The inter-assay CVs were 7.8% for E_2, 3.6% for testosterone, 6.5% for androstenedione, and 10.4% for P_4. The cross reactivity with other hormones for each type of kit was negligible.

Quantitative real-time polymerase chain reaction (qPCR): Total RNA was isolated from frozen follicles using the RNeasy Micro Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. RNA was treated with DNAse to remove any possible genomic DNA contamination. The concentration of RNA in each sample was measured at 260 nm using the Nanodrop ND1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). The cDNA was synthesized by reverse transcribing the mRNA (200 ng) using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Then cDNA was diluted to 1:4 with nuclease free water.

qPCR was conducted using CFX96 Real-time System C1000 Thermal Cycler (Bio-Rad). All samples were measured in triplicate, and each reaction contained 2 µL of diluted cDNA, 0.66 µL (330nM) of gene-specific primers (Integrated DNA Technologies, Inc, Coralville, IA, Table 1), 2.34 µL of nuclease-free water, and 5 µL of SsoFast EvaGreen Supermix (Bio-Rad) for a final volume of 10 µL. The qPCR program consisted of an enzyme activation step (95 °C for 1 min), an amplification and quantification program [40 cycles of 95 °C for 10 sec, annealing/extension for 10 sec (Table 1), single fluorescence reading], a 72 °C for 5 min step, a melt curve (65 - 95°C heating 0.5 °C per second with continuous fluorescence readings) and a final step at 72 °C for 5 min.

Primer sequences are shown in Table 1. The specificity of each primer was tested using BLASTN2.218+ and by the presence of a single peak in the melt curve analysis. In addition, each product was run on 2% agarose gels to confirm the product size. A standard curve was generated from six serial dilutions of one of the samples (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64); thus, allowing analysis of the amount of cDNA in the exponential phase. β-Actin was used as a reference gene. Final values were expressed as genomic equivalents and were calculated as the ratio of each gene to β-actin. The reported data were obtained from the mean expression values of 3-4 separate culture experiments.

Statistical analysis: All data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). For all comparisons, statistical significance was assigned at $p \leq 0.05$. Comparisons between DMSO and the different doses of MXC were conducted on data obtained from 3 to 4 experiments using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or a test for linear regression when applicable.

3.4 Results

Effect of MXC on steroid hormone levels

Previous studies have shown that MXC inhibits growth of mouse antral follicles after 96 h of in vitro culture (Gupta *et al*., 2006; Miller *et al*., 2005). In the present study, we confirmed these previous findings and showed that MXC inhibits follicle growth at 48, 72, and 96 h (Figure 3.1). We further investigated whether MXC alters E_2 levels. At 24h, MXC did not alter E_2 levels. However, at 48 (MXC 1, 10 and 100 µg/ml) and 96 h (MXC 10 and 100 µg/ml), MXC exposure significantly decreased E_2 levels compared to vehicle control (DMSO) (Figure 3.2).

In addition to E_2 levels, we also investigated whether MXC affected biosynthetic hormone precursors of E_2 such as testosterone, androstenedione, and P_4. MXC did not affect testosterone levels at 24 and 48 h, but it decreased the levels at 96 h (MXC 10 and 100 µg/ml) compared to controls (Figure 3.3). MXC did not affect androstenedione levels at 24 h, but it decreased the levels at 48 (MXC 1, 10, and 100 µg/ml) and 96 h (MXC 1, 10, and 100 µg/ml) compared to controls (Figure 3.4). MXC did not alter P_4 levels significantly at any time point, however, the test for linearity using regression analysis showed that P_4 levels trended downwards at 96 h compared to DMSO controls ($p=0.065$) (Figure 3.5).

Effect of MXC on expression of enzymes that synthesize ovarian steroids

Since MXC decreased levels of steroid hormone precursors and E_2 levels, we next examined whether the decreased steroidogenesis could be due to effects of MXC on mRNA expression of steroidogenic enzymes. We focused on expression levels because previous studies have shown that the activities of enzymes are directly related to their transcriptional regulation (Jones and DeCherney, 2005; Miller, 1989).

MXC significantly affected expression of *Cyp19a1* levels, the enzyme that converts testosterone to E_2 (Fig. 6). At 24 h (MXC 1 µg/ml), MXC significantly increased expression of *Cyp19a1* enzyme levels compared to DMSO. However, at 48 (MXC 100 µg/ml) and 96 h (MXC 10 and 100 µg/ml), MXC significantly decreased expression of *Cyp19a1* compared to DMSO. In the MXC 1 µg/ml treatment group, the enzyme levels were significantly decreased at 48 and 96 h compared to 24 h (Figure 3.6).

MXC significantly decreased levels of *Hsd17b1,* the enzyme that converts androstenedione to testosterone (Fig. 7). In the MXC 100 µg/ml treatment groups, *Hsd17b1* expression levels were significantly decreased at 24, 48, and 96 h compared to DMSO groups. In the MXC 1 µg/ml group, the *Hsd17b1* enzyme levels were significantly decreased at 96 h compared to 24 h (Figure 3.7).

MXC also significantly decreased *Cyp17a1* levels, the enzyme that converts P_4 to androstenedione (Fig. 8). These enzyme expression levels were significantly decreased at 48 h (MXC 10 and 100 µg/ml) and showed a significantly decreased trend at 96 h compared to DMSO. The levels in the MXC 10 µg/ml group were significantly decreased at the 48 and 96 h time points compared to 24 h (Figure 3.8).

MXC significantly decreased *Hsd3b1* levels, the enzyme that converts pregnenolone to P_4 and dehydroepiandrosterone to androstenedione (Fig. 9). *Hsd3b1* expression levels were significantly decreased as early as 24 h (MXC 100 µg/ml) and continued to be decreased at 48 (MXC 100 µg/ml) and 96 h (MXC 10 and 100 µg/ml) compared to DMSO groups. In the MXC 10 µg/ml treatment group, *Hsd3b1* expression levels were significantly different across different time points. The levels were significantly decreased at 48 and 96 h compared to 24 h. In the

MXC 100 µg/ml treatment group, *Hsd3b1* expression levels were significantly decreased at 48 h compared to 24 h (Figure 3.9).

MXC significantly decreased *Cyp11a1* expression levels, the mitochondrial enzyme that converts cholesterol to pregnenolone (Fig. 10). *Cyp11a1* expression levels were significantly decreased at 48 (MXC 100 µg/ml) and 96 h (MXC 10 and 100 µg/ml) compared to DMSO groups. There were no differences between expression levels in each treatment group when compared across different points (Figure 3.10).

MXC significantly decreased *Star* expression levels, the enzyme that is involved in the transport of cholesterol from the cytoplasm into mitochondria during steroidogenesis in the theca cells (Fig. 11). When treatment groups were compared within each time point, *Star* expression levels were significantly increased at 48 h (MXC 100 µg/ml) and significantly decreased at 96 h with MXC 10 µg/ml (p=0.01) and trended toward decreased expression with MXC 100 µg/ml (p=0.065) compared to DMSO. When the expression levels of each treatment group were compared across the different time points, *Star* expression levels in DMSO and MXC 1 µg/ml treatment groups were increased at 96 h compared to 24 h (Figure 3.11).

Effect of MXC on expression of an enzyme that metabolize E_2

MXC significantly altered expression of *Cyp1b1* levels, the enzyme that converts E_2 to catecholestrogen. Specifically, MXC (100 µg/ml) significantly reduced expression of *Cyp1b1* levels by 24 h and did not affect the levels at 48 h, but significantly induced expression levels by 96 h compared to DMSO controls (Figure 3.12). In the MXC 10 µg/ml treatment group, expression levels were increased at 48 h compared to 24 h and in the MXC 100 µg/ml treatment group, expression levels were increased at 96 h compared to 24 h. The ability of MXC to

increase expression of *Cyp1b1* at 96 h was similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD: 10nM), a known inducer of *Cyp1b1* expression (Dasmahapatra *et al*., 2001).

3.5 Discussion

The present studies were designed to test the hypothesis that MXC decreases E_2 levels produced by antral follicles by altering steroidogenic and metabolic enzymes. This study confirms results from previous studies that MXC inhibits follicle growth in cultured mouse antral follicles (Gupta *et al*., 2006; Miller *et al*., 2005). This study also expands previous studies by examining the effects of MXC on the steroidogenesis pathway. In the current study, MXC decreased follicle growth, hormone levels, and most enzyme mRNA levels as early as 48 h. However, MXC decreased *Hsd17b1* and *Hsd3b1* expression levels as early as 24 h. Thus, this study shows that some of the earliest induced changes by MXC may be in some steroidogenic enzymes. These early changes in *Hsd17b1* and *Hsd3b1* may lead to the decrease in hormone levels observed in MXC-treated follicles. Further, since adequate levels of sex steroids are required for follicle growth (Drummond *et al.,* 2006), the changes in hormones may contribute to MXC-induced inhibition of follicle growth.

Our data showed that MXC decreases E_2 levels in cultured mouse antral follicles. These results are consistent with previous findings performed in rat ovarian culture studies (Cummings and Laskey, 1993) and studies on bovine granulosa cells showing that MXC (1 μg/ml) decreases E_2 levels (Tiemann *et al*., 1996). However, the present study demonstrates that MXC disrupts E_2 produced by intact mouse antral follicles and expands previous studies by showing that MXC disrupts steroid precursors made in both thecal and granulosa cells of the follicle. The data obtained using intact antral follicles are significant since antral follicles are the functional units

of the ovary that produce sex steroid hormones, which are involved in various important physiological functions in the body, including ovulation.

The present studies also showed that MXC inhibits testosterone production and causes a decreased trend in P_4 levels in cultured mouse antral follicles. These data are consistent with previous studies in rat ovarian cultures showing that MXC decreases testosterone, but not P_4 levels in the media (Cummings and Laskey, 1993) and that MXC decreases P_4 levels in vivo (Chapin *et al*., 1997). Our data on P_4 levels are also similar to data from studies on porcine granulosa cells showing that MXC decreases P_4 levels (Chedrese and Feyles, 2001). In addition, another study in cultured bovine granulosa cells showed that MXC decreases P_4 synthesis (Tiemann *et al*., 1996). Other studies have also shown that MXC decreases testosterone levels in male rat testes in vivo (Murono *et al*., 2006). Thus, our data in conjunction with others from rat, porcine, and bovine studies suggest that MXC may have similar effects on steroidogenesis across various tissues and species. The present study also expands previous studies by measuring another steroid hormone - androstenedione, which also is significantly decreased in response to MXC. Thus, suggesting that in addition to E_2, testosterone, and P_4, MXC has significant negative effects on androstenedione levels.

Some of the effects of MXC on steroid levels are similar to those produced by metabolites of MXC. Previous studies in liver cells have shown that MXC is metabolized to metabolites such as 2-(p-hydroxyphenyl)-2-(p-methoxyphenyl)-1,1,1-trichloroethane (mono-OH MXC) and 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Kapoor *et al*., 1970) by cytochrome P450 (Cyp) enzymes *Cyp1a2* and *Cyp2c29* (Stresser and Kupfer, 1998). These Cyp enzymes are expressed in the ovary and can be altered by MXC treatment (Paulose *et al*., 2011; Symonds *et al*., 2006). Thus, the ovary is equipped with *Cyp* enzymes to metabolize MXC to

mono-OH MXC and HPTE. Mono-OH MXC has been shown to decrease E_2, androgen, and P_4 levels by altering the expression of steroidogenic enzymes in the antral follicles from mouse ovaries (Craig *et al*., 2010) and HPTE been shown to inhibit P_4 and E_2 production in rat theca-interstitial and granulosa cultures by inhibiting steroidogenic enzymes (Akgul *et al*., 2008; Zachow and Uzumcu, 2006). This raises the possibility that the effects of MXC on steroid levels observed in this study are due to MXC metabolites and not just MXC alone. It is possible that ovarian follicles have the capacity to convert MXC to mono-OH MXC and/or HPTE and that these metabolites inhibit steroid hormone levels. Alternatively, it is possible that the ovarian follicles do not convert MXC to its metabolites and that MXC itself can inhibit steroid levels. Future studies should distinguish between these possibilities.

Since we observed alterations in hormone levels in response to MXC, we investigated the mechanism by which MXC alters hormone levels by measuring steroidogenic and metabolic enzymes and found that MXC does indeed decrease steroidogenic enzymes and increase the metabolic enzyme, *Cyp1b1*. Some of the changes in metabolic enzymes may explain our data on follicle growth and steroid levels. At 48 h, MXC 1 μg/ml inhibits E_2 levels, but these levels return to normal at 96 h. MXC also increases expression of *Cyp19a1* at 24 h. Thus, we speculate that MXC increases *Cyp19a1* protein and activity and that this in turn leads to an increase in conversion of testosterone to E_2, which restores E_2 levels. Because E_2 is important for follicle growth, the normal E_2 levels could then restore follicle growth at the 1 μg/ml of MXC.

While there are limited other data on the effects of MXC on steroidogenic enzymes, a few studies have shown that metabolites of MXC may affect steroidogenic enzymes in other tissues and culture systems. Studies in rat granulosa cells showed that the MXC metabolite, HPTE (10 μM; 48 h), blocks FSH-stimulated mRNA expression levels of *Cyp19a1* and that

these levels remain suppressed in the presence of dibutyryl-cAMP (db-cAMP) (Zachow and Uzumcu, 2006). Another metabolite of MXC, mono-OH MXC, also inhibits *Cyp19a1* mRNA expression levels in mouse antral follicles (Craig *et al*., 2010). Studies in human and rat testes indicate that MXC and HPTE decrease HSD17B1 activity (Hu *et al*., 2010; Vaithinathan *et al*., 2008) and studies in mouse antral follicles show that mono-OH MXC decreases expression of *Cyp17a1* levels (Craig *et al*., 2010). The previous studies have shown that MXC and its metabolite HPTE inhibit HSD3B1 activity in human and rat testes (Hu *et al*., 2010; Vaithinathan *et al*., 2008). Studies on rat ovaries showed that in-vivo exposure to MXC (100 mg/kg) significantly reduces luteinizing hormone receptor expression in large antral follicles and significantly reduces CYP11A1 immunoreactivity in the ovaries (Armenti *et al*., 2008). In addition, another study in rat Leydig cells showed that HPTE decreases testosterone production by inhibiting the mRNA levels of *Cyp11a1* (Akingbemi *et al*., 2000). Another study in rat testis showed that MXC reduces STAR protein levels (Vaithinathan *et al*., 2008). In the present study, MXC up-regulated the *Star* mRNA expression levels transiently at 48 h and inhibited *Star* expression by 96 h. A similar increase in *Star* levels was previously reported in granulosa cells at 48 h in response to HPTE in the presence of db-cAMP (Zachow and Uzumcu, 2006). These data suggest that MXC may affect STAR expression differently in the testes and ovary. In the ovary, it is possible that *Star* overexpression is an attempt to increase the transfer of cholesterol to the mitochondria to increase substrate for *Cyp11a1* and compensate for inhibition of hormonal levels present downstream in the steroidogenic pathway.

In addition to E_2 biosynthetic enzymes, E_2 metabolic enzymes such as Cyp1a1 and Cyp1b1 are important in maintenance of E_2 levels. TCDD is the classical halogenated aromatic hyrdrocarbon that has been shown to induce Cyp1a1 and Cyp1b1 enzymes in different tissues,

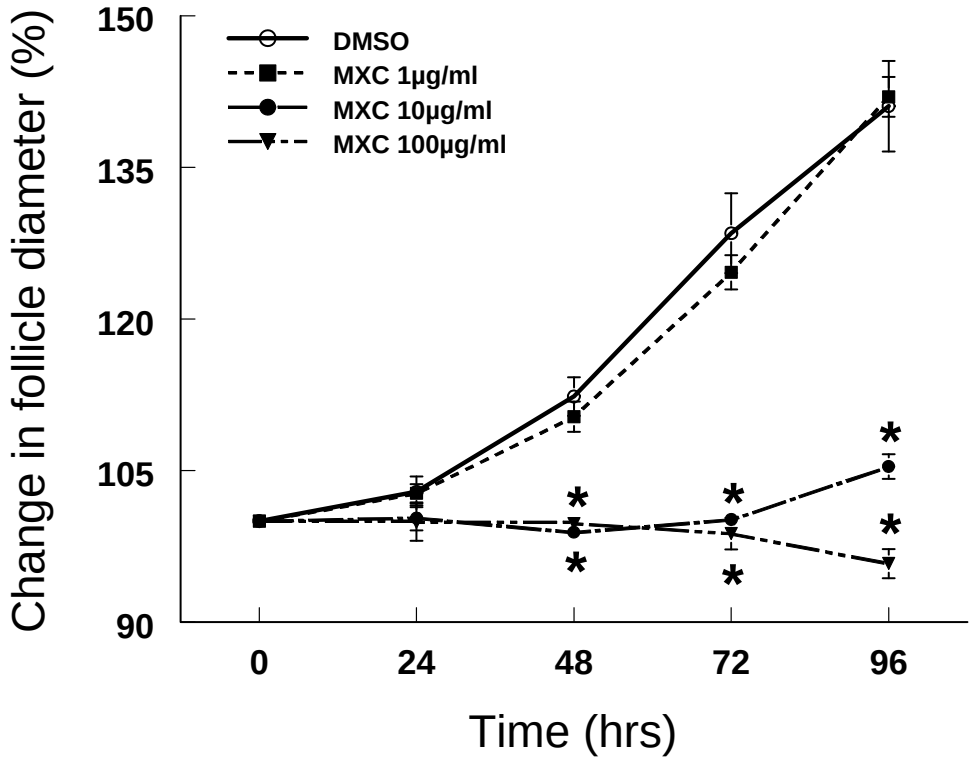
including granulosa cells of the ovary (Bofinger *et al*., 2001; Dasmahapatra *et al*., 2001; Whitlock, 1993). Cyp1a1 and Cyp1b1 are involved in C-2 and C-4 hydroxylations of E_2 respectively, leading to formation of catecholestrogens (Spink *et al*., 1992, 1994). In the present study, levels of *Cyp1b1* were measured as they are shown to be expressed constitutively at higher levels compared to *Cyp1a1* in the ovary (Dasmahapatra *et al*., 2001). *Cyp1b1* levels were reduced at 24 h (MXC 100 μg/ml) and reached normal levels by 48 h, but significantly increased at 96 h to levels similar to those produced by TCDD (10nM). At 24 h, MXC did not alter the expression of steroidogenic enzymes, but it decreased expression of metabolic enzymes, resulting in no change in steroidogenesis and leading to no change in E_2 levels. At 48 h, MXC inhibited steroidogenic enzymes, but not metabolic enzymes, resulting in decreased steroidogenesis with no changes in E_2 metabolism, which finally lead to decreased E_2 levels. At 96 h, MXC decreased steroidogenic enzymes and increased metabolic enzymes, resulting in decreased synthesis and increased clearance of E_2, thus leading to further decrease in E_2 levels. Thus, these data show that both steroidogenesis and metabolism of E_2 were affected by MXC over time.

Collectively, our results show that MXC decreases steroidogenic enzymes and increases metabolic enzymes (*Cyp1b1*) in mouse antral follicles. The reduction in steroidogenic enzymes likely leads to decreased synthesis of androgens and E_2. Further, increase in the metabolic enzyme *Cyp1b1* likely increases metabolism of E_2 in follicles. Together, with decreased availability of hormone precursors and increased expression of metabolic enzyme *Cyp1b1*, MXC causes significant decrease in E_2 levels and these low levels of steroids may contribute to MXC-induced inhibition of follicle growth. The reasons for all these MXC-induced changes are unknown, but could include one or more common factors upstream of enzyme expression. MXC

as well as its metabolites have been shown to bind both nuclear and membrane estrogen receptors. Thus, it is possible that MXC binds to estrogen receptors and that this leads to changes in gene expression, which eventually leads to changes in steroidogenesis and follicle growth. Future studies will determine whether this is the case.

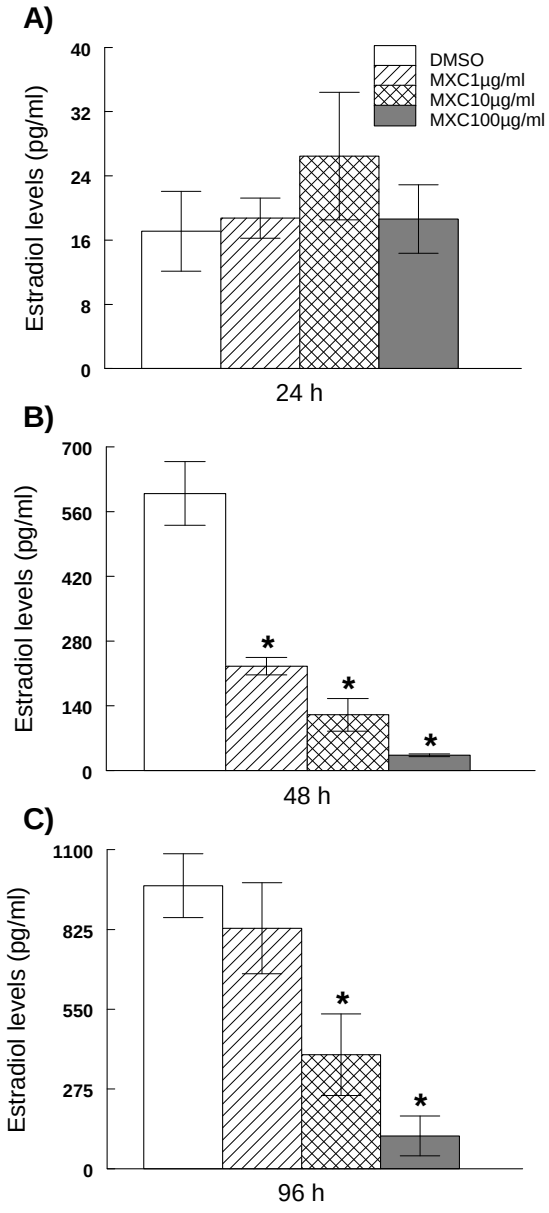
3.6 Figures and Legends

Figure 3.1 Effect of in vitro MXC exposure on antral follicular growth



Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) for 96 h. Growth of follicles was monitored during culture, recorded in µm and reported as percentage change. Data represent means ± SE from 3 separate experiments [* indicates significant difference from controls *(n= 12-16 follicles per treatment; p≤0.05)]*.

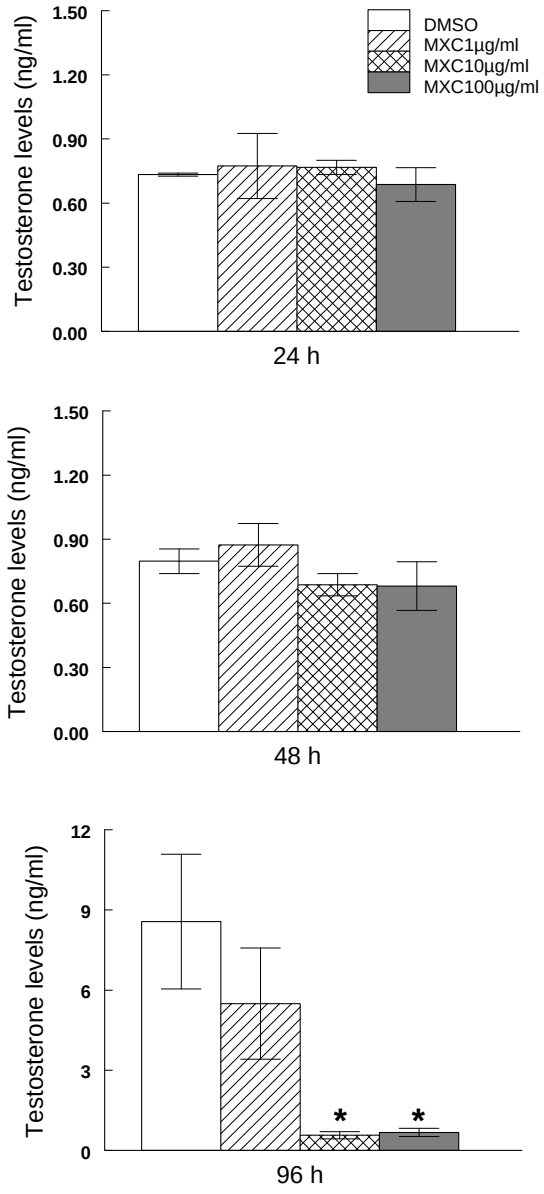
Figure 3.2 Effect of in vitro MXC exposure on estradiol (E_2) levels



Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. The media were subjected to measurements of E_2 levels by enzyme-linked immunosorbent assay (ELISA). **(A)** E_2 levels at 24 h **(B)** E_2 levels at 48 h **(C)** E_2 levels at 96 h. Data represent means ± SEM from 3 separate

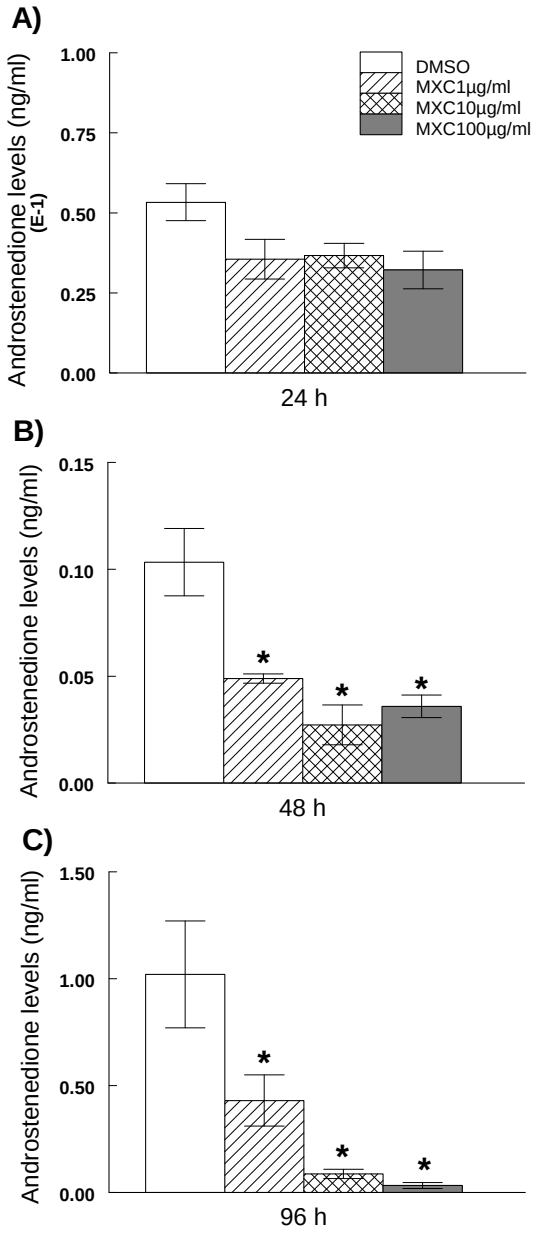
experiments [* indicates significant difference from controls *(n=9-12 follicles per treatment; p≤0.05)]*.

Figure 3.3 Effect of in vitro MXC exposure on testosterone levels



Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. The media were subjected to measurements of testosterone levels by ELISA. **(A)** testosterone levels at 24 h **(B)** testosterone levels at 48 h **(C)** testosterone levels at 96 h. Data represent means ± SEM from 3 separate experiments [* indicates significant difference from controls *(n=9-12 follicles per treatment; p≤0.05)]*.

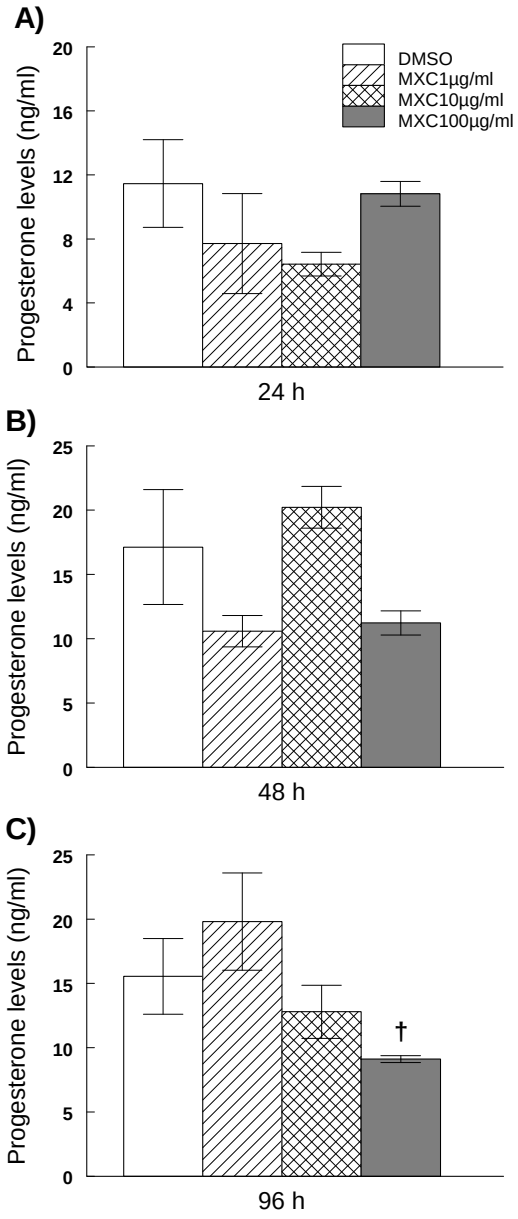
Figure 3.4 Effect of in vitro MXC exposure on androstenedione levels



Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. The media were subjected to measurements of androstenedione levels by ELISA. **(A)** androstenedione levels at 24 h **(B)** androstenedione levels at 48 h **(C)** androstenedione levels at 96 h. Data represent means ± SEM

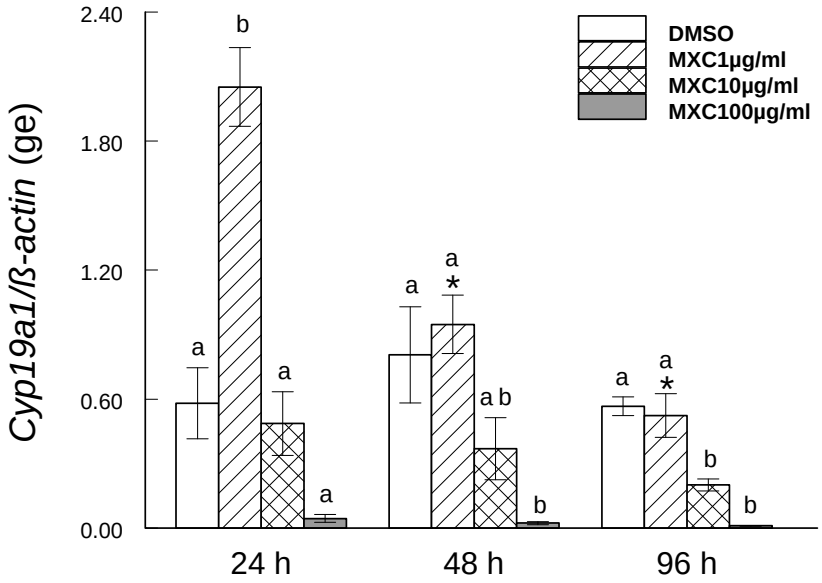
from 3 separate experiments [* indicates significant difference from controls *(n=9-12 follicles per treatment; p≤0.05)]*.

Figure 3.5 Effect of in vitro MXC exposure on progesterone (P_4) levels



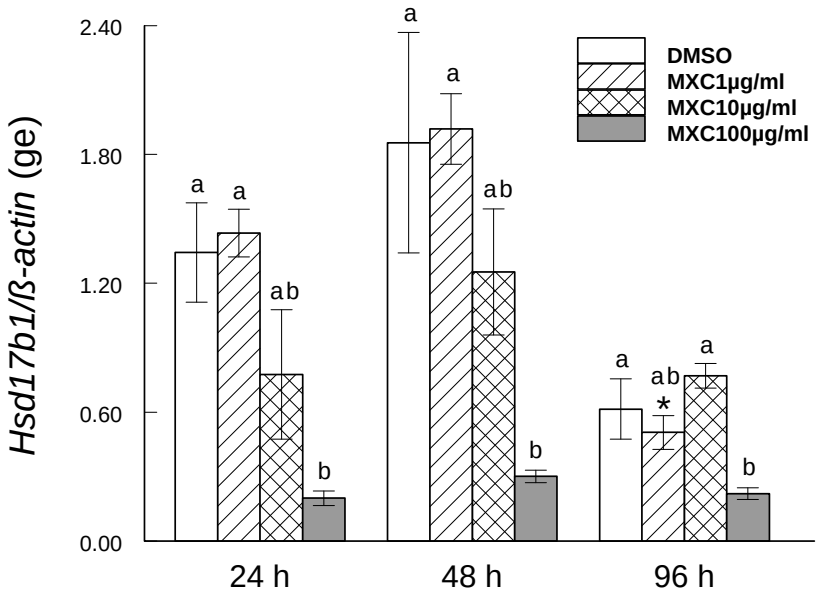
Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. The media were subjected to measurements of *P_4* levels by ELISA. **(A)** P_4 levels at 24 h **(B)** P_4 levels at 48 h **(C)** P_4 levels at 96 h. Data represent means ± SEM from 3 separate experiments [† *$p=0.065$* using linear regression *(n=9-12 follicles per treatment)]*.

Figure 3.6 Aromatase (*Cyp19a1*) levels in response to MXC



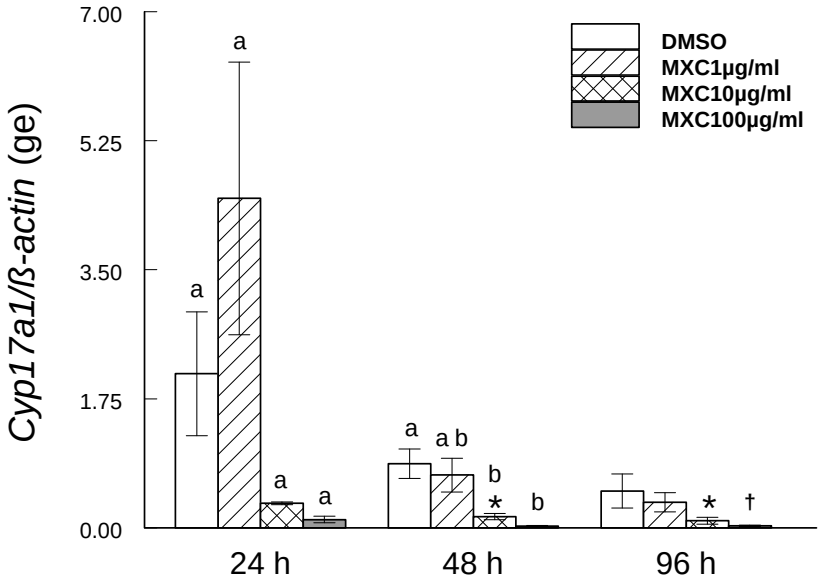
Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. *Cyp19a1* mRNA expression was measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments [n=12-14 follicles per treatment; *$p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Figure 3.7 17β-hydroxysteroid dehydrogenase (*Hsd17b1*) levels in response to MXC



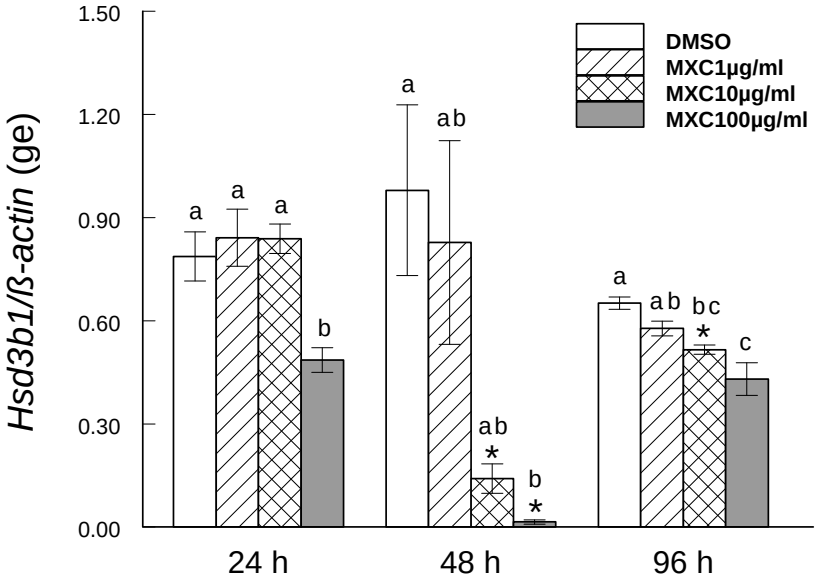
Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. *Hsd17b1* mRNA expression was measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments [n=12-14 follicles per treatment; *$p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Figure 3.8 17α-hydroxylase/17,20-lyase (*Cyp17a1*) levels in response to MXC



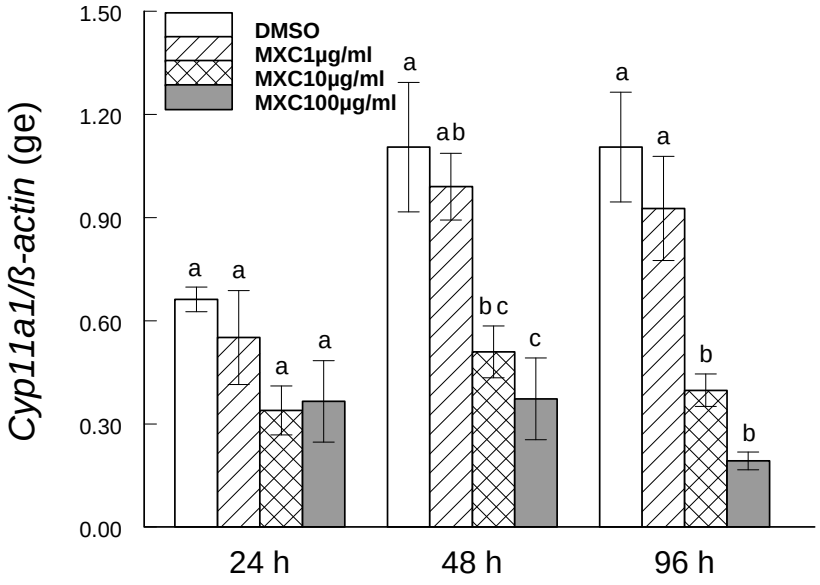
Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. *Cyp17a1* mRNA expression was measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments [n=12-14 follicles per treatment; *$p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point; † $p \leq 0.05$ using linear regression].

Figure 3.9 3β-hydroxysteroid dehydrogenase (*Hsd3b1*) levels in response to MXC



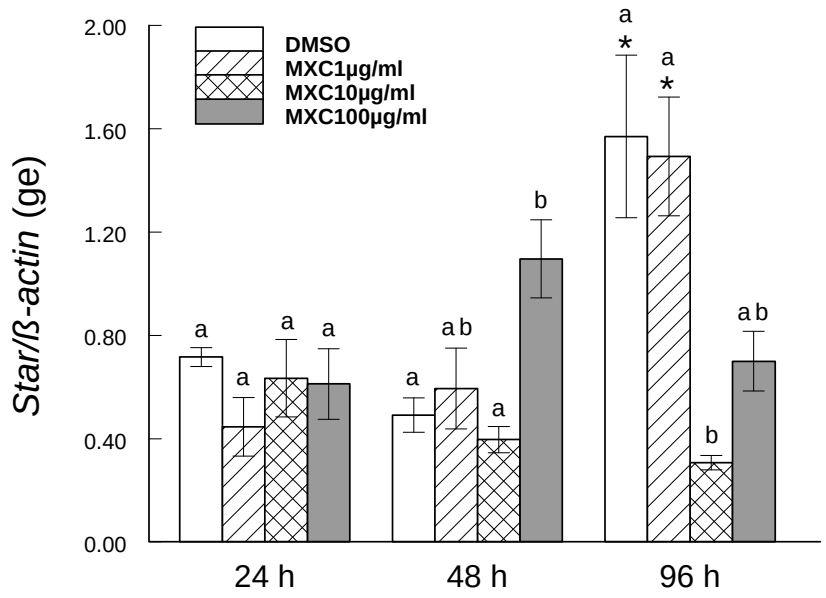
Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. *Hsd3b1* mRNA expression was measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments [n=12-14 follicles per treatment; *$p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Figure 3.10 Cholesterol side chain cleavage enzyme (*Cyp11a1*) levels in response to MXC



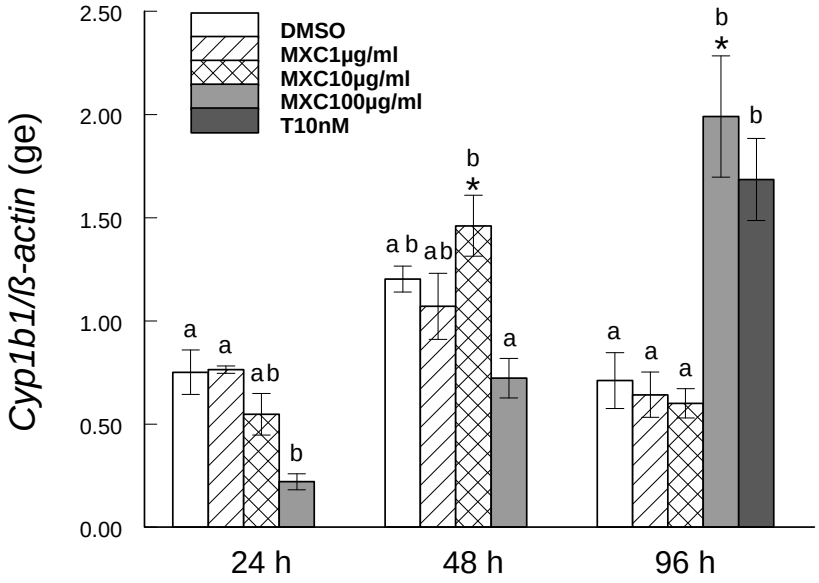
Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. *Cyp11a1* mRNA expression was measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments [n=12-14 follicles per treatment; Bars with different letters are significantly different from each other within each time point].

Figure 3.11 Steroidogenic acute regulatory protein (*Star*) in response to MXC



Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. *Star* mRNA expression was measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments [n=12-14 follicles per treatment; *$p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Figure 3.12 Cytochrome P450 1b1 (*Cyp1b1*) levels in response to MXC



Antral follicles isolated mechanically from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, and 96 h. *Cyp1b1* mRNA expression was measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments [n=12-14 follicles per treatment; *$p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

3.7 Table Real-time qPCR primer accession numbers, abbreviations, and sequences.

Accession no.	Gene name	Abbreviation	Annealing/ extension temperature (^{0}C)	Forward	Reverse
NM_007393.3	*actin, beta*	*Actb*	57.3	5'-GGGCACAGTGTGGGTGAC- 3'	5'-CTGGCACCACACCTTCTAC- 3'
NM_009994.1	*cytochrome P450, family 1, subfamily b, polypeptide 1*	*Cyp1b1*	60	5'-GCGACGATTCCTCCGGGCTG- 3'	5'-TGCACGCGGGCCTGAACATC- 3'
NM_019779.3	*cytochrome P450, family 11, subfamily a, polypeptide 1*	*Cyp11a1*	60	5'-AGATCCCTTCCCCTGGTGACAATG- 3'	5'-CGCATGAGAAGAGTATCGACGCATC- 3'
NM_007809.3	*cytochrome P450, family 17, subfamily a, polypeptide 1*	*Cyp17a1*	56.2	5'-CCAGGACCCAAGTGTGTTCT- 3'	5'-CCTGATACGAAGCACTTCTCG- 3'
NM_007810.3	*cytochrome P450, family 19, subfamily a, polypeptide 1*	*Cyp19a1*	55.3	5'-CATGGTCCCGGAAACTGTGA- 3'	5'-GTAGTAGTTGCAGGCACTTC- 3'
NM_008293.3	*hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1*	*Hsd3b1*	59.4	5'-CAGGAGAAAGAACTGCAGGAGGTC- 3'	5'-GCACACTTGCTTGAACACAGGC- 3'
NM_010475.1	*hydroxysteroid (17-beta) dehydrogenase 1*	*Hsd17b1*	58	5'-ACTGTGCCAGCAAGTTTGCG- 3'	5'-AAGCGGTTCGTGGAGAAGTAG- 3'
NM_011485.4	*steroidogenic acute regulatory protein*	*Star*	57.5	5'-CAGGGAGAGGTGGCTATGCA- 3'	5'-CCGTGTCTTTTCCAATCCTCTG- 3'

3.8 References

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CHAPTER IV

Methoxychlor induces atresia by altering Bcl2 factors and inducing caspase activity

4.1 Abstract

The organochlorine pesticide methoxychlor (MXC) is an endocrine disrupting chemical that has been shown to affect reproductive functions in adult females by causing persistent estrous cyclicity, reduced fertility, and ovarian atrophy. MXC is widely used in many countries to prevent and kill various species of insects that attack field crops, trees, vegetables, fruits, gardens, stored grain, livestock, and domestic pets. MXC reduces fertility by increasing atresia of antral follicles and by decreasing numbers of healthy antral follicles in adult female mice. However, the mechanism by which MXC causes follicular atresia is unknown. Previous studies have shown that MXC causes atresia at 96 h, in part, by increasing the pro-apoptotic factor *Bax* and decreasing the anti-apoptotic factor *Bcl2* in antral follicles. Further, *Bcl2* overexpression and *Bax* deletion help to protect antral follicles from MXC-induced atresia. Presently, the earliest time at which MXC induces atresia is unknown and whether it alters other pro-apoptotic and anti-apoptotic factors in the antral follicles is also unknown. Thus, this work was designed to test the hypothesis that MXC induces atresia at early time points and that it also alters other pro-apoptotic (*Bok* and *Casp3*) and anti-apoptotic factors (*Bcl-xL*) in addition to *Bcl2* and *Bax*. In addition, we also hypothesized that MXC alters caspase activity in the follicles. To test this hypothesis, antral follicles were mechanically isolated from ovaries of CD1 female mice aged 35-40 days. The isolated antral follicles (10-15 per treatment) were cultured in supplemented α-minimum essential media in the presence of

vehicle control (dimethylsulfoxide; DMSO) or MXC (1, 10, 100 μg/ml) for 24, 48, and 96 hours at 37^0C and 5%CO_2. At selected times, follicles were subjected to histological analysis of atresia or collected and snap frozen. RNA was extracted from frozen follicles, cDNA was synthesized and then subjected to quantitative real-time PCR for the measurement of mRNA levels of *Bax*, *Bcl2*, *Bcl-xL*, *Bok*, and *Casp3*. The results indicate that at 24 h, MXC decreased *Bok* and *Bcl-xL* mRNA levels ($p \leq 0.05$, $n=3$) and increased *Bax* mRNA levels ($p \leq 0.05$, $n=3$). MXC did not affect *Casp3* and *Bcl2* mRNA levels at this time point. At 48 h, MXC decreased *Casp3* mRNA levels ($p \leq 0.05$, $n=3$), but did not affect expression of the other pro-apoptotic and anti-apoptotic genes. At 96 h, MXC significantly increased expression of *Bcl2*, *Bcl-xL*, and *Bax* mRNA levels ($p \leq 0.05$, $n=3$), but did not affect *Bok* and *Casp3* mRNA levels. Caspase activities were increased at all time points. We also found that MXC exposure increases *Bax* as early as 24 h, but it does not induce changes in *Bcl2* expression at this time point. This increase in *Bax/Bcl2* ratio at 24 h, activates Casp3/7 activities which may lead to morphological atresia beginning at 48 h. Collectively, these results show that MXC alters both pro-apoptotic factors and anti-apoptotic factors in cultured antral follicles. These data suggest that MXC may induce atresia by altering the balance between several pro-apoptotic and anti-apoptotic factors in antral follicles.

4.2 Introduction

The ovaries of female mammals contain a fixed number of primordial follicles at the time of birth (Hirshfield, 1991). These primordial follicles grow into more mature follicle stages known as primary, pre-antral, and antral follicles. During the reproductive lifespan, a constant stream of follicles grows from the primordial to the antral stage. Antral follicles are the only type

of follicles capable of releasing a fertilizable oocyte and they are the major sites of synthesis of steroid hormones such as estrogens. Although a few antral follicles ovulate and become fertilized, between 95-99% of all follicles die via an apoptotic process known as atresia.

The endocrine disrupting chemical methoxychlor (MXC) is an organochlorine pesticide, which is primarily used against various species of insects that attack field crops, trees, vegetables, fruits, gardens, stored grain, livestock and domestic pets (ATSDR, 2002). MXC specifically targets antral follicles in the ovary and its exposure leads to adverse reproductive functions in adult female mice by causing persistent estrus, ovarian atrophy, and follicular atresia (Eroschenko *et al*., 1997; Martinez and Swartz, 1991). Exposure to MXC during pregnancy in female mice revealed a significant increase in atretic follicles in female litters. A residual effect of MXC was observed as a significant advance in the time of vaginal opening in the second generation of litters (Swartz and Corkern, 1992). In vivo studies in mice have shown that MXC causes antral follicle toxicity, characterized by an increased percentage of atretic follicles and a decreased number of healthy antral follicles compared to controls (Borgeest *et al*., 2002). Many in vitro studies also have shown that MXC inhibits follicle growth and increases atresia of cultured mouse antral follicles (Gupta *et al*., 2006; Miller *et al*., 2005).

Previous studies have only determined that MXC causes atresia of antral follicles after chronic (96 h) exposure in vitro. However, they have not determined if acute MXC exposure causes atresia nor have they determined the time course of onset of atresia with MXC exposure. Such information is critical for understanding the effects of MXC on ovarian follicles and for delineating the mechanism involved in MXC induced atresia. Thus, the current study examined both acute (24 - 48 h) and chronic (96 h) exposure to MXC to determine the earliest time point at which MXC induces atresia.

Despite the potential adverse reproductive abnormalities associated with MXC exposure, little is also known about the mechanisms by which MXC induces atresia in antral follicles. The present study was designed to identify whether the mechanism by which MXC destroys antral follicles involves Bcl_2 family members and caspases. Bcl_2 family members include two distinct groups: pro-apoptotic factors that promote apoptosis (e.g., Bax, Bok) and anti-apoptotic factors that promote cell survival (e.g., Bcl_2, Bcl-xL) (Hsu and Hsueh, 2000; White, 1996). The fate of follicles depends on the balance between these different pro-apoptotic and anti-apoptotic factors. Bcl_2 and Bax in particular are major regulators of follicular atresia in the mammalian ovary (Flaws *et al*., 2001; Hsu *et al*., 1996). There are many functions of Bcl_2 family members, but their primary roles are to regulate mitochondrial membrane homeostasis and the release of cytochrome c from the mitochondrial intermembrane space, both of which are decisive steps in apoptosis. Pro-apoptotic factors such as Bax dimerize with the anti-apoptotic factor Bcl_2; thus relieving the apoptotic protease activating factor-1 (Apaf-1) from suppression by Bcl_2 proteins and promoting caspase activation. Additionally, these pro-apoptotic factors alter mitochondrial membrane homeostasis and increase cytochrome c release by forming pores in the outer mitochondrial membrane and opening the permeability transition pore following mitochondrial permeability transition, while anti-apoptotic factors such as Bcl_2 counteract these actions. Cytochrome c interacts with Apaf-1 and procaspase 9 to form an apoptosome. Apoptosome formation results in activation of caspase 9 and subsequent activation of downstream caspases, which in turn results in DNA fragmentation (Hsu and Hsueh, 2000). Given the roles of pro-apoptotic and anti-apoptotic factors in regulating atresia, the proposed work was designed to test the hypothesis that MXC induces atresia by altering pro-apoptotic and anti-apoptotic factors in the antral follicles of the ovary. Specifically, we examined the effects of MXC on expression of

Bcl$_2$, *Bcl-xL*, *Bax*, *Bok*, and caspase levels and activity after both acute and chronic MXC exposure.

4.3 Materials and Methods

***Chemicals*:** MXC (99% pure) was purchased from Chemservice (West Chester, PA). Stock solutions of MXC for in vitro experiments were prepared using vehicle control (diemthylsulfoxide; DMSO) (Sigma, St. Louis MO) as a solvent, and in various concentrations (2, 20, and 200 mg/ml) that permitted an equal volume of solvent to be added to individual culture wells for each treatment group. Thus, final concentrations of MXC in culture were 1, 10, and 100 μg/ml (ppm). The doses used in these experiments were selected based on previously published studies showing that these concentrations of MXC induce toxicity in antral follicles, granulosa cell culture models, and in uterine leiomyoma cells (Chedrese and Feyles, 2001; Gupta *et al*., 2006; Miller *et al*., 2005). The selected doses are environmentally relevant. Environmental levels of MXC range from 40–160 ppm in waters downstream of areas exposed to MXC (Wallner *et al*., 1969) to 4 ng/kg/day in humans exposed through food products (ATSDR, 2002). These concentrations are also relevant to occupational exposure levels. Normally, serum levels of MXC are reported to be below the level of detection. However, a study involving an occupational exposure in farm workers has shown that MXC concentrations in serum can reach up to 5.16 μg/ml (ATSDR, 2002). Thus, the occupational exposure dose is much higher than normal human exposure and lies between the doses used in the present experiments. For controls and MXC treatment groups, DMSO was used at 0.05%, which is able to solubilize MXC in aqueous media. DMSO, ITS (insulin, transferrin, selenium), penicillin, and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). Alpha-minimal essential media (α-MEM) was

obtained from Invitrogen (Carlsbad, CA). Human recombinant follicle stimulating hormone (rFSH) was obtained from Dr. A.F. Parlow from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

***Animals*:** Adult female cycling CD-1 mice were purchased from Charles River Laboratories (Charles River, CA) and housed in the core animal facility located at College of Veterinary Medicine, University of Illinois and maintained on 12L:12D cycles. Mice were housed in the animal facility for at least two days to relieve transportation stress, given *ad libitum* food and water, and temperature was maintained at 22±1 °C. Animals were euthanized at 35-39 days of age by carbon dioxide (CO_2) inhalation followed by cervical dislocation. The ovaries were removed and antral follicles were isolated as explained below. The University of Illinois Institutional Animal Care and Use Committee approved all protocols involving animal care, euthanasia, and tissue collection.

***Antral follicle culture*:** Ovaries were removed and antral follicles were isolated from the ovaries of mice between 35-39 days old because this time point was used in previous studies, and this is the age at which mice are cycling young adults (Borgeest *et al*., 2004; Basavarajappa *et al*., 2011). Antral follicles were isolated mechanically from the ovaries based on relative size and interstitial tissue was removed using fine watch-maker forceps. About 3-4 mice were used per experiment and they yielded approximately 20-30 follicles per mouse. Once follicles were isolated, they were placed individually in wells of a 96-well culture plate with 150 µl of unsupplemented α-MEM prior to treatment. A dose response regimen of MXC (1-100 µg/ml)

and vehicle controls was individually prepared in supplemented α-MEM. For treatment, unsupplemented α-MEM was removed from each well and replaced with 150 µl of supplemented α-MEM containing MXC or DMSO. The follicles were cultured in supplemented media according to previously described protocols (Basavarajappa *et al*., 2011). Follicles then were incubated for 24, 48, and 96 hours (h). Non-treated controls (supplemented medium alone) were used in each experiment as a control for culture conditions. At the end of 24, 48, and 96 h follicle cultures, follicles were collected, snap frozen, and stored at -80^{o}C for later use. Follicle cultures were repeated three times to obtain enough power for statistical analysis.

***Histological evaluation of atresia*:** At selected times in culture, follicles were collected in Dietrick's solution and processed through series of washes with ethanol: 70% for 10 min, 85% for 10 min, 95% for 7 min (2X), and 100% for 7 min (2X). The follicles were embedded in plastic blocks using Technovit 7100 kits from Heraeus Kulzer GmbH, Germany. The follicles were incubated in a pre-infiltration medium for one hour and then with infiltration medium overnight before they were embedded in plastic blocks using embedding medium. Pre-infiltration solution was prepared with one part 100% ethanol and one part base solution. Infiltration solution was prepared with 1 gm of hardener-1 with 100 ml of base solution. Embedding medium was prepared by adding 1 ml of hardener-2 to 15 ml of infiltration solution.

The follicles embedded in plastic blocks were sectioned into 2 µm sections using a microtome. Follicle sections were stained with Lee's methylene blue-basic fuchsin stain for 30 sec and washed with distilled water before they were cover slipped. Each follicle section was examined for level of atresia (follicle death) as evidenced by the presence of apoptotic bodies and reported at the highest level observed throughout the tissue. Follicles were rated on a scale of

1-4 for the presence of apoptotic bodies: 1= healthy, 2 = less than 10% apoptotic bodies (early atresia), 3 = 11-30% apoptotic bodies (mid atresia), 4 = greater than 30% apoptotic bodies (late atresia) as previously described by Miller et al. (2005). Ratings were averaged and plotted to compare the effect of chemical treatments on atresia levels.

***Quantitative real-time polymerase chain reaction (qPCR)*:** Total RNA was isolated from frozen follicles using the RNeasy Micro Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. RNA was treated with DNAse to remove any possible genomic DNA contamination. The concentration of RNA in each sample was measured at 260 nm using the Nanodrop ND1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). The cDNA was synthesized by reverse transcribing mRNA (50 ng) using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The cDNA was diluted to 1:2 with nuclease free water.

qPCR was conducted using a CFX96 Real-time System C1000 Thermal Cycler (Bio-Rad). All samples were measured in triplicate, and each reaction contained 2 µL of diluted cDNA, 0.6 µL (300nM) of gene-specific primers (Integrated DNA Technologies, Inc, Coralville, IA, Table 1), 2.4 µL of nuclease-free water, and 5 µL of SsoFast EvaGreen Supermix (Bio-Rad) for a final volume of 10 µL. The qPCR program consisted of an enzyme activation step (95 °C for 1 min), an amplification and quantification program [40 cycles of 95 °C for 10 sec, 60 °C annealing/extension for 10 sec, single fluorescence reading], a 72 °C for 5 min step, a melt curve (65 - 95 °C heating 0.5 °C per second with continuous fluorescence readings) and a final step at 72 °C for 5 min.

Primer sequences are shown in Table 1. The specificity of each primer was tested using BLASTN2.218+ and by the presence of a single peak in the melt curve analysis. In addition, each product was run on 2% agarose gels to confirm the product size. A standard curve was generated from six serial dilutions of one of the samples (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64); thus, allowing analysis of the amount of cDNA in the exponential phase. β-Actin was used as a reference gene. Final values were expressed as genomic equivalents and were calculated as the ratio of each gene to β-actin. The reported data were obtained from the mean expression values of 3-4 separate culture experiments.

***Caspase activity assay*:** Caspase activity in the cultured antral follicles was measured using the Caspase-Glo 3/7 Assay Kit from Promega. The follicles were cultured for 24, 48, and 96 h and then transferred with 100 µl of medium to a white-walled 96 well plate. Caspase-Glo 3/7 assay reagent (100 µl) was added and the plate was incubated at room temperature for 150 min. The caspase-3/7 present in the follicles cleaves the luminogenic substrate containing the DEVD sequence, releasing aminoluciferin, which in turn acts as a substrate for luciferase emitting luminescence. The amount of luminescence is a direct measurement of the caspase activity. The luminescence was captured using Synergy 2 Alpha Microplate Reader (Biotek).

***Statistical analysis*:** All data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). For all comparisons, statistical significance was assigned at $p \leq 0.05$. Comparisons between DMSO and the different doses of MXC were conducted on data obtained from 3 to 4 separate experiments using one-way analysis of variance (ANOVA) or Kruskal-Wallis test followed by Tukey's post hoc test or a test for linear regression or Mann-Whitney test when applicable.

4.4 Results

Effect of MXC on atresia

Previous studies have shown that MXC induces atresia of mouse antral follicles at 96 h of in vitro culture (Gupta *et al*., 2006; Miller *et al*., 2005). In the present study, we confirmed these previous findings by showing that MXC induces atresia at 96 h (MXC 100 μg/ml) (Fig.1). We also expanded previous studies by determining the earliest time at which MXC induces atresia. At 24 h, MXC did not induce atresia, but by 48 h, MXC (1, 10 and 100 μg/ml) increased follicle atresia ratings compared to vehicle control (DMSO) (Figure 4.1).

Effect of MXC on expression of pro-apoptotic and anti-apoptotic factors

Since MXC induced atresia in antral follicles, we next examined whether the induction of atresia could be due to effects of MXC on mRNA expression of pro-apoptotic and anti-apoptotic factors. Upon treatment with MXC, the mRNA expression levels of the pro-apoptotic factor *Bax* were increased at all time points. MXC (100 μg/ml) significantly increased expression of *Bax* levels at 24, 48, and 96 h compared to DMSO (Figure 4.2). Interestingly, *Bax* levels were increased as early as 24 h, preceding the time of onset of morphological atresia.

MXC did not affect *Bcl2* levels at 24 and 48 h time points, but, MXC (100 μg/ml) significantly increased expression of *Bcl2* at 96 h compared to DMSO (Figure 4.3). Interestingly, there were no changes in this anti-apoptotic factor at 24 h, the time point which precedes morphological atresia.

From the mRNA expression values of *Bax* and *Bcl2*, we calculated the *Bax/Bcl2* ratio across all time points. On treatment with MXC (100 μg/ml), *Bax/Bcl2* ratios were increased at 24

h, not altered at 48 h, and decreased by 96 h (Figure 4.4). Thus, the *Bax/Bcl2* ratio was increased as early as 24 h, preceding the onset of morphological atresia.

Since MXC altered *Bax*, *Bcl2*, and their ratios, we wanted to measure the expression of other factors involved in induction of apoptosis in the antral follicles of the ovary. We found that MXC altered levels of another anti-apoptotic factor *Bcl-xL*. MXC significantly increased *Bcl-xL* levels at 24 and 96 h compared to DMSO (Figure 4.5). However, MXC did not affect *Bcl-xL* levels at 48 h. MXC exposure decreased levels of the pro-apoptotic factor *Bok*. At 24 h, MXC significantly decreased *Bok* levels compared to DMSO. However, MXC did not affect *Bok* levels at the 48 and 96 h time points (Figure 4.6).

Since increased Bax/Bcl2 ratios are associated with induction of caspase activities (Basu and Haldar, 1998), we examined the levels of both *Casp3* mRNA as well as Casp3 activities in antral follicles exposed to vehicle and MXC. We found that MXC treatment significantly decreased *Casp3* levels at 48 h compared to DMSO, but it did not affect *Casp3* levels at 24 and 96 h. (Figure 4.7).

Effect of MXC on CASPASE3/7 *activity*

While MXC decreased mRNA expression levels of *Casp3* at 48 h, it did not change *Casp3* mRNA levels at a time that precedes the morphological onset of atresia. Studies, however, have shown that changes in caspase activities are important for induction of atresia rather than changes in mRNA levels. Thus, we measured the CASP3/7 activities in cultured antral follicles exposed to controls or MXC and found that MXC significantly increases CASP3/7 activities. Specifically, MXC (100 μg/ml) significantly increases CASP3/7 activities at 24, 48, and 96 h compared to DMSO (Figure 4.8).

4.5 Discussion

The present studies were conducted to determine if acute exposure to MXC induces atresia and if MXC induces atresia of antral follicles by altering pro-apoptotic and anti-apoptotic factors. First, we examined the effects of both acute and chronic exposure to MXC on antral follicles. We found that MXC induces atresia as early as 48 h and that it continues to do so for up to 96 h. These findings are consistent with previous studies indicating that MXC increases atresia of mouse antral follicles at 96 h of culture (Miller *et al*., 2005). However, these studies also expand previous studies by showing that MXC induces atresia as early as 48 h. This suggests that acute exposure to MXC can quickly set into motion processes that result in death of antral follicles and that once the death process is initiated, it is not possible to stop it.

Given that MXC induces atresia in antral follicles, we next examined the effect of MXC on *Bax* and *Bcl2* expression in follicles, as it is well known from previous studies that *Bax* and *Bcl2* are important regulators of atresia in the ovary. Previous studies have shown that Bax deficient mice contain fewer atretic follicles than WT mice (Perez *et al*., 1999). Similarly, another study has shown that Bcl2 overexpression increases the number of primordial follicles at birth, possibly due to decreased apoptosis (Flaws *et al*., 2001). Further, mice deficient in Bcl2 have an increased number of unhealthy primordial follicles and an increased number of apoptotic oocytes (Ratts *et al*., 1995). In addition to being important regulators of natural atresia, Bax and Bcl2 are involved in chemical induction of apoptosis. The polyaromatic hydrocarbon dimethylbenz(*a*)anthracene has been shown to increase Bax levels, inducing apoptosis in mouse oocytes (Matikainen *et al*., 2001). Another chemical, 4-vinylcyclohexene diepoxide (VCD) also has been shown to induce atresia in small pre-antral follicles by altering Bcl2 family members

(Hu *et al*., 2001). Further, chromium has been shown to increase Bax and decrease Bcl2, inducing apoptosis in mouse ovarian granulosa cells (Banu *et al*., 2011).

We found that MXC exposure increases *Bax* as early as 24 h, but does not induce changes in *Bcl2* expression at this time point. This increases the *Bax/Bcl2* ratio at 24 h, which may lead to morphological atresia beginning at 48 h. We also found that MXC increases *Bax* (48 and 96 h), increases *Bcl2* (96 h) and decreases in *Bax/Bcl2* ratios (96 h) at later time points. This may be an attempt by the follicles to rescue themselves from MXC-induced atresia. However, our data indicate that once MXC induces atresia at 48 h, the follicles cannot recover from atresia throughout the culture. It is likely that any increase in anti-apoptotic factor Bcl2 cannot compensate for pro-apoptotic effects of Bax. The changes we observed with Bax and Bcl2 in response to MXC in vitro are consistent with previous in vivo studies showing that chronic exposure (20 days) to MXC induces atresia of antral follicles by increasing Bax and without altering Bcl2 levels in the mouse ovary (Borgeest *et al*., 2004). The data are also consistent with previous in vitro studies showing that MXC increases *Bax* (48 h) and decreases *Bcl2* (96 h) expression (Miller *et al*., 2005).

Given that the pro-apoptotic factor Bax plays a decisive role in MXC-induced atresia, we wanted to expand our findings by determining whether other pro- and anti-apoptotic factors are involved in MXC-induced atresia. Previous studies have shown that *Bcl-xL* acts as anti-apoptotic factor by preventing apoptosis in the ovary (Tilly *et al*., 1995). Hence, the current studies measured *Bcl-xL* expression levels in response to MXC in the antral follicles of the ovary. Our data indicate that MXC increases *Bcl-xL* levels at 24 and 96 h. However, these changes in *Bcl-xL* expression are not consistent with the time course for onset of MXC-induced atresia. Thus, it is likely that *Bcl-xL* factors are not involved in MXC-induced atresia.

We also examined the effect of MXC on the pro-apoptotic factor Bok. This factor has been shown to induce apoptosis in reproductive tissues (Hsu *et al*., 1997). Hence, the current studies measured *Bok* expression in response to MXC in the ovary. Our data indicate that MXC decreased *Bok* levels at 24 h, but did not affect *Bok* expression at other time points. These, changes in *Bok* at 24 h are not consistent with the morphological appearance of atresia that occurred at 48 h. Thus, it is possible that *Bok* is not involved in MXC-induced atresia. It is possible that the decrease in *Bok* levels at 24 h is an attempt by the follicles to rescue themselves from atresia. However, attempts were not successful in rescuing atresia evident at 48 h and remains throughout culture.

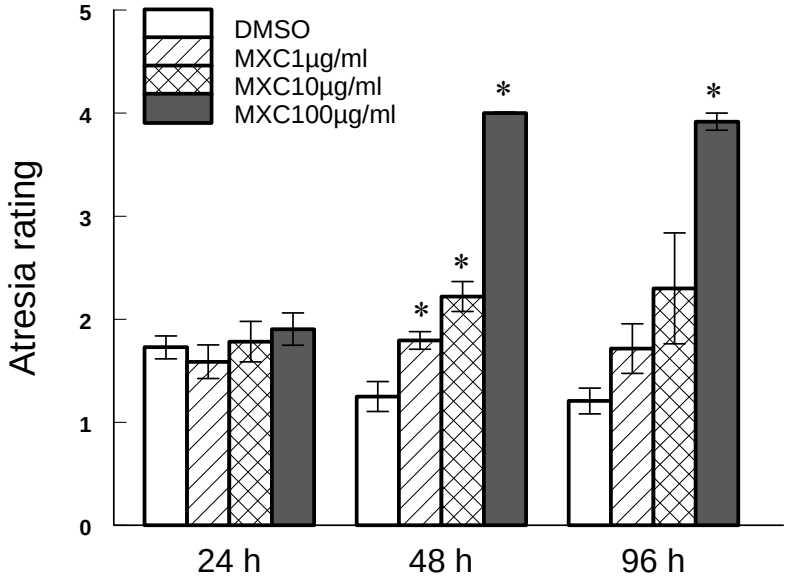
While changes in Bcl2 family members help regulate apoptosis, caspase3 is the critical rate limiting factor in the apoptotic pathway. In apoptotic cells, pro-caspase3 is cleaved and activated by Casp9, the activated Casp3 then autoregulates to increase the permeability of mitochondrial membrane by conversion of anti-apoptotic factors to pro-apoptotic factors, further amplifying Casp3 activity. Finally, Casp3 cleaves several proteins and DNA leading to formation of apoptotic bodies (Cheng *et al*., 1997). In the present study, we measured *Casp3* mRNA and Casp3/7 activities in MXC treated follicles. Our data indicate that MXC decreases *Casp3* mRNA levels at 48 h in the follicles. In contrast, MXC increases Casp3/7 activities at all the time points in the follicles. These data suggest that MXC-induced atresia is not regulated by changes in *Casp3* mRNA expression. Instead, MXC-induced atresia is regulated by increase in Casp3 activity. While to our knowledge, no studies have examined the effects of MXC on caspase activities in the ovary, some studies have shown that other chemicals induce Casp3 activity or protein levels in the ovarian cells. Several studies in granulosa cells have shown that acute exposure to molybdenum and chromium induce apoptosis by inducing Casp3 (Banu *et al*., 2011;

Kolesarova *et al*., 2010). Other in vivo studies have shown that chronic exposure to VCD induces apoptosis in pre-antral follicles by inducing Casp3 (Thompson *et al*., 2005; Devine *et al*., 2002). Our data are consistent with these studies in that chemicals including MXC cause apoptosis by inducing Casp3 activity in follicles or granulosa cells.

In conclusion, the present studies provide evidence that acute exposure to MXC induces atresia as quickly as 48 h. The present studies also show that MXC exposure alters expression of key anti- and pro-apoptotic factors and increases caspase activity. At 24 h, MXC increases *Bax* levels and does not affect *Bcl2* levels. This increases the *Bax/Bcl2* ratio, which in turn may increase the mitochondrial permeability leading to activation of Casp3/7 activities. Thus, these early changes in *Bax* expression and Casp3/7 activity induce onset of morphological atresia beginning at 48 h. Once MXC-induced atresia occurs, the follicles cannot recover and continue to undergo atresia at 96 h (Fig.9).

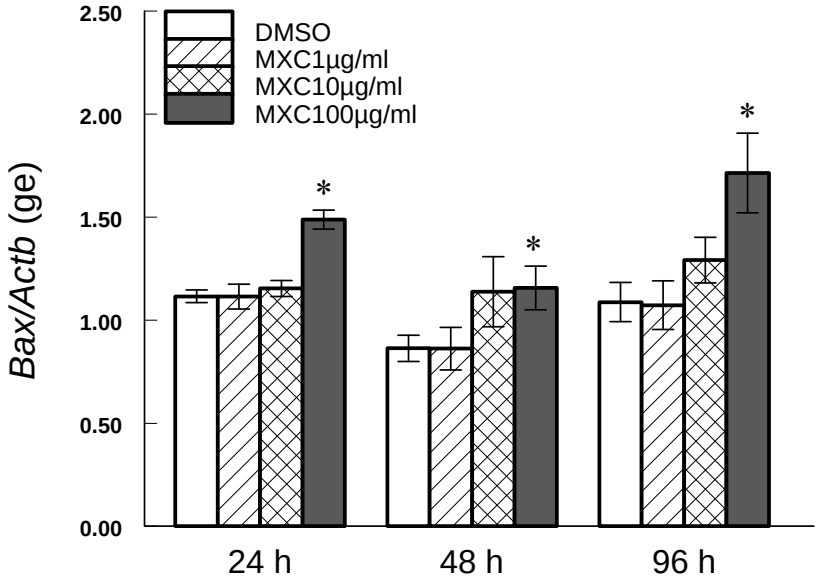
4.6 Figures and Legends

Figure 4.1 Effect of in vitro MXC exposure on antral follicular atresia



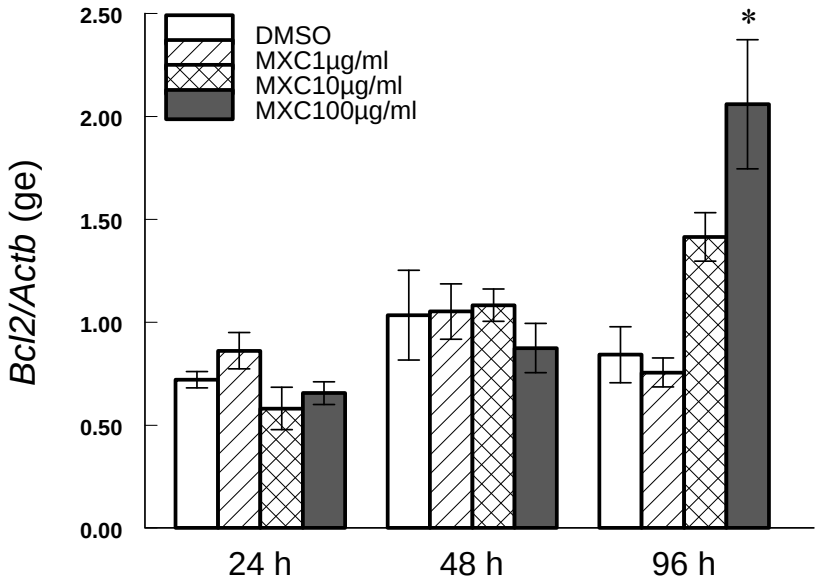
Antral follicles isolated from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) for 24, 48, or 96 h. At the end of culture, follicles were subjected to histological analysis of atresia. Data represent means ± SEM from 3 separate experiments (* indicates significant difference from vehicle controls; n= 4 - 6 follicles per treatment; $p \leq 0.05$).

Figure 4.2 *Bax* levels in response to MXC



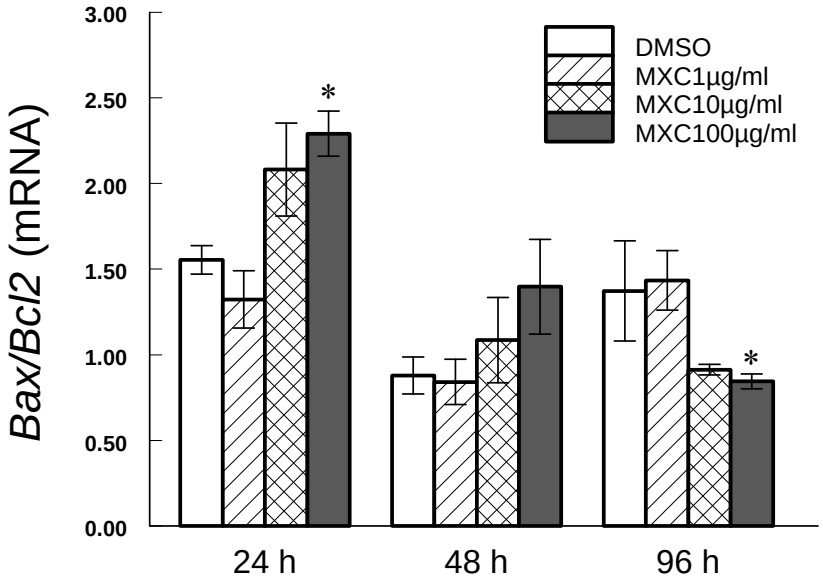
Antral follicles isolated from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, or 96 h. *Bax* mRNA levels were measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments (* indicates significant difference from vehicle controls; n=12-14 follicles per treatment; $p \leq 0.05$).

Figure 4.3 *Bcl2* levels in response to MXC



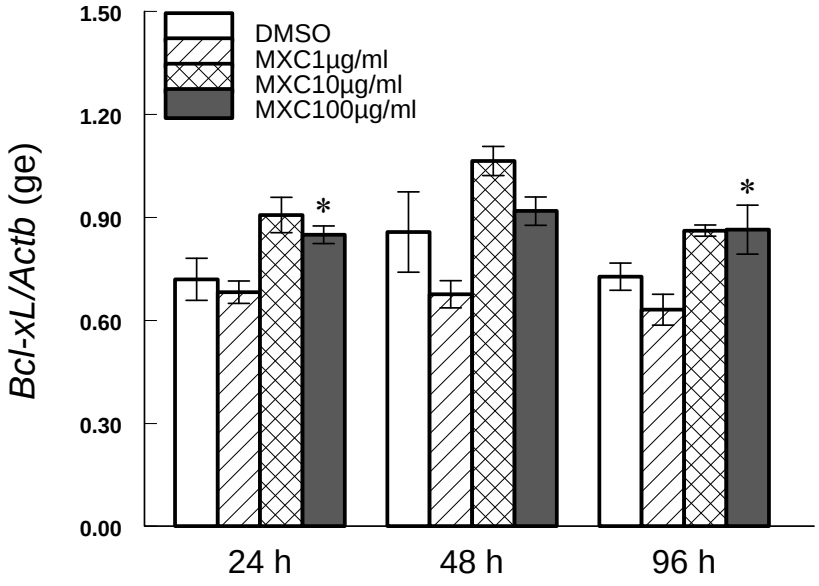
Antral follicles isolated from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, or 96 h. *Bcl2* mRNA levels were measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments (* indicates significant difference from vehicle controls; n=12-14 follicles per treatment; $p \leq 0.05$).

Figure 4.4 *Bax/Bcl2* ratios in response to MXC



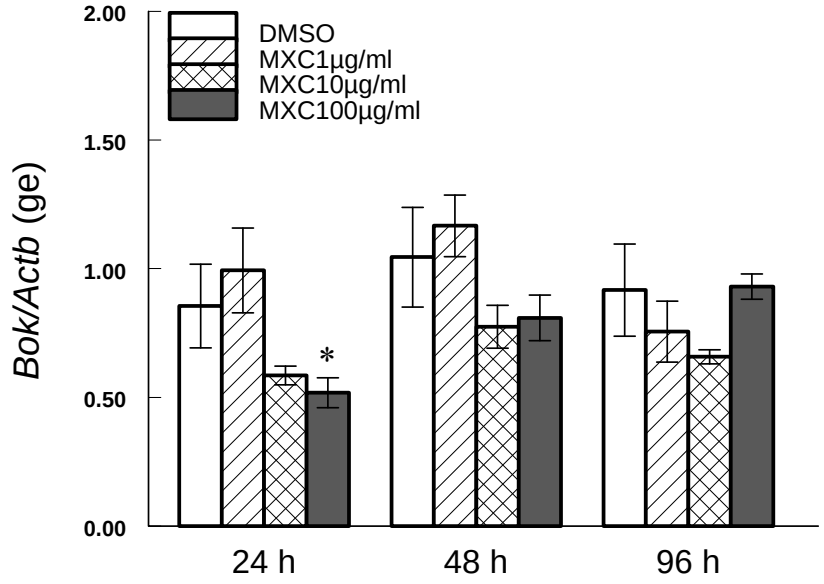
Antral follicles isolated from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, or 96 h. Using qPCR, mRNA levels of *Bax* and *Bcl2* were measured in antral follicles and *Bax/Bcl2* ratios were calculated. Data represent means ± SEM from 3 separate experiments (* indicates significant difference from vehicle controls; n=12-14 follicles per treatment; $p \leq 0.05$).

Figure 4.5 *Bcl-xL* levels in response to MXC



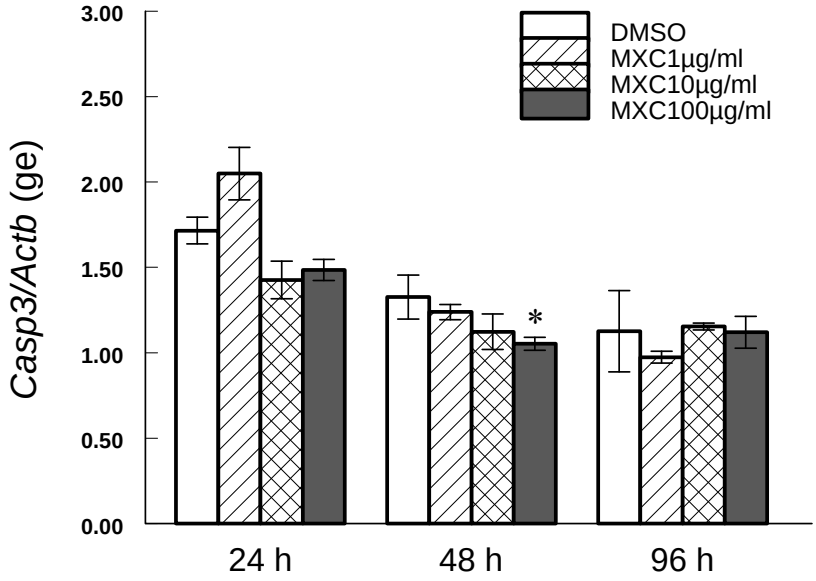
Antral follicles isolated from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, or 96 h. *Bcl-xL* mRNA levels were measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments (* indicates significant difference from vehicle controls; n=12-14 follicles per treatment; $p \leq 0.05$).

Figure 4.6 *Bok* levels in response to MXC



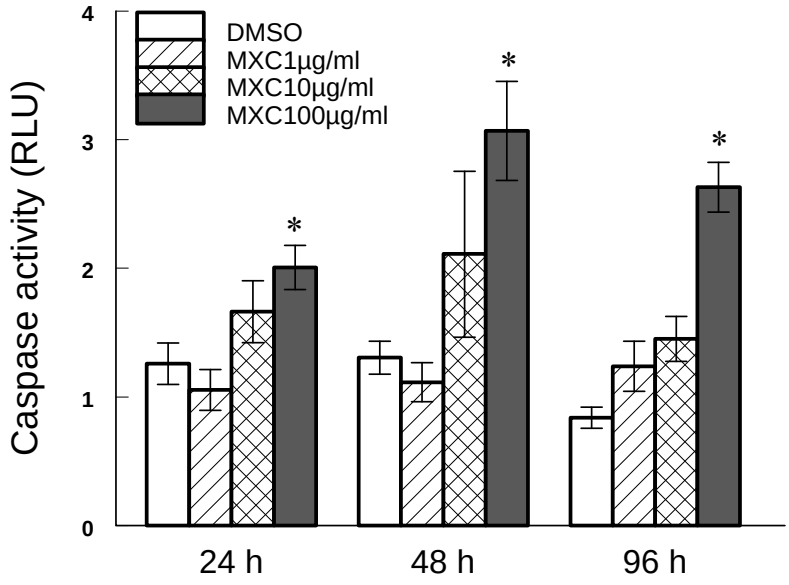
Antral follicles isolated from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, or 96 h. *Bok* mRNA levels were measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments (* indicates significant difference from vehicle controls; n=12-14 follicles per treatment; $p \leq 0.05$).

Figure 4.7 *Casp3* levels in response to MXC



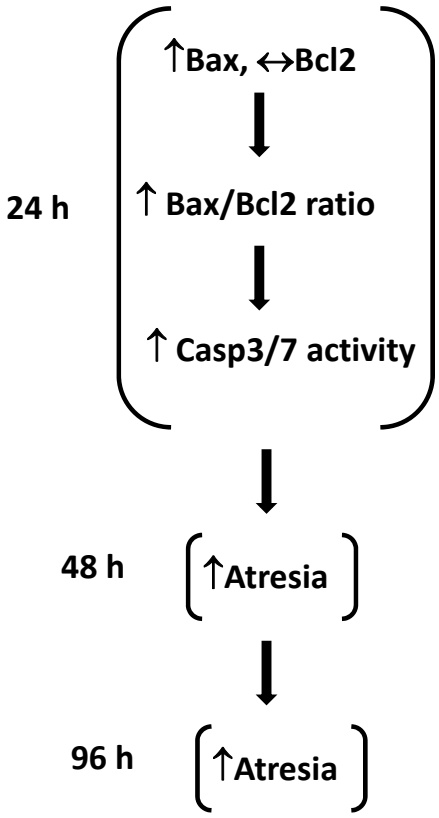
Antral follicles isolated from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, or 96 h. *Casp3* mRNA levels were measured in antral follicles using qPCR. Data represent means ± SEM from 3 separate experiments (* indicates significant difference from vehicle controls; n=12-14 follicles per treatment; $p \leq 0.05$).

Figure 4.8 Casp3/7 activities in response to MXC



Antral follicles isolated from CD-1 mice were exposed to vehicle control (DMSO) or MXC (1-100 µg/ml) and cultured for 24, 48, or 96 h. At the end of culture, follicles were subjected to measurement of *Casp3/7* activities. Data represent means ± SEM from 3 separate experiments (* indicates significant difference from vehicle control; n=12-14 follicles per treatment; $p \leq 0.05$).

Figure 4.9 Model of MXC-induced atresia



Exposure to MXC at 24 h increases the *Bax* expression and does not alter *Bcl2* expression, leading to an increased *Bax/Bcl2* ratio. This increase in *Bax/Bcl2* ratio leads to increase in Casp3/7 activity, which finally induces atresia at 48 h. Once atresia occurs, follicles fail to revive and continue to remain atretic at 96 h.

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CHAPTER V

Methoxychlor inhibits growth and induces atresia through aryl hydrocarbon receptor pathway in mouse ovarian antral follicles

5.1 Abstract

Methoxychlor (MXC) is an organochlorine pesticide used against pests and insects that attack crops, gardens, vegetables, pets, and livestock. MXC has been shown to reduce fertility, and to cause persistent estrus and ovarian atrophy. Further, MXC inhibits growth and induces atresia of mouse antral follicles in vitro. Little is known, however, about the mechanisms by which MXC causes slow growth and atresia of antral follicles. Several studies indicate that many chemicals act through the aryl hydrocarbon receptor (AHR) pathway and one study has shown that MXC binds to the AHR in liver cells. Thus, we hypothesized that MXC induces antral follicle toxicity through an AHR mediated pathway. The current study tested the hypothesis that MXC binds to AHR to inhibit follicle growth and induce atresia of antral follicles. To test binding of MXC to AHR, we transfected granulosa cells with xenobiotic response element based reporter plasmids and found that MXC binds to AHR. To confirm whether MXC acts through the AHR pathway, antral follicles were surgically isolated from ovaries of C57BL/6 female wild-type (WT) and AHR null (AHRKO) mice aged 30-35 days. The isolated antral follicles (10-15 per treatment) were cultured in vehicle control (dimethylsulfoxide; DMSO) or MXC (1, 10, 100 μg/ml) for 168 h at 37ºC and 5%CO_2 in α-minimum essential media. During culture, follicle growth was monitored every 24 h by measuring follicular diameter on two perpendicular axes. After culture, the follicles were

subjected to histological analysis of atresia. The results indicate that MXC (10, 100 μg/ml) significantly inhibits follicular growth in wild-type antral follicles by 168 h. The results also indicate that MXC (10, 100 μg/ml) significantly induced atresia in WT antral follicles. Conversely, MXC (10 μg/ml) did not significantly inhibit follicle growth or induce atresia in AHRKO follicles. These data indicate that AHR deletion protects antral follicles against MXC induced slow growth and atresia. Collectively, these data show that MXC acts through the AHR pathway to inhibit follicle growth and induce atresia in antral follicles of the ovary.

5.2 Introduction

Endocrine disrupting chemicals (EDCs) such as pesticides, plasticizers, cosmetics, solvents, paints and pollutants are present in the environment. EDCs exert their toxicity by affecting the synthesis, secretion, transport, binding, action, and elimination of variety of hormones present in the body (Crisp *et al*., 1998). The EDC methoxychlor (MXC) is an organochlorine pesticide that is widely used in many parts of the world, primarily to prevent or destroy insects or pests that feed on field crops, gardens, fruits, vegetables, stored grain and domestic animals (ATSDR, 2002). MXC gained popularity in 1970s and replaced the potent chlorinated pesticide dichlorodiphenyltrichloroethane (DDT) after its usage was restricted in the US and other parts of the world. MXC was extensively used in the farming industry until it was banned in 2004 because of failure of registration with the EPA (Stuchal *et al*., 2006). Globally, humans and domestic animals are exposed to MXC through extensive usage of this chemical and in the US, through imported agricultural products.

MXC has been shown to induce persistent vaginal estrus, decrease ovarian weights, and increased atresia of large follicles in adult female mice (Martinez and Swartz, 1991). Exposure to

MXC in utero increases atretic follicles in F1a litters and the residual effect of MXC induces premature vaginal opening in F1b litters. In addition, mothers exposed to MXC have an increased gestation period and increased number of dead fetuses compared to vehicle controls during the first pregnancy (Swartz and Corkern, 1992). Neonatal exposure to MXC also induces ovarian atrophy, decreases relative ovarian weight, and decreases corpora lutea numbers (Eroschenko *et al*., 1995). Collectively, these previous studies indicate that MXC targets the ovary and affects fertility in laboratory rodents.

More recent studies indicate that MXC damages the ovary by inhibiting antral follicle growth and increasing atresia. In vivo studies in mice indicate that MXC specifically targets antral follicles by increasing the number of atretic antral follicles and decreasing the percentage of healthy antral follicles in the ovary (Borgeest *et al*., 2002). Several in vitro studies have also shown that MXC inhibits growth and increases atresia of mouse antral follicles (Miller *et al*., 2005; Gupta *et al*., 2006).

While it is known that MXC inhibits growth and increases atresia of antral follicles, the mechanism by which it does so is unclear. Many studies indicate that other EDCs such as polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and polyhalogenated biphenyls mediate their toxicity through the aryl hydrocarbon receptor (AHR) pathway (Okey *et al*., 1994; Poland *et al*., 1976). However, limited information is available about whether MXC works via the AHR pathway. One study by Han et al., (2007) suggests that MXC may bind to AHR, inhibiting the action of 2,3,4,8-tetrachlorodibenzo-p-dioxin (TCDD) in murine Hepa-1c1c7 cells (Han *et al*., 2007). Thus, the current study was designed to determine the involvement of the AHR pathway in mediating the effects of MXC treatment on antral follicles

of the mouse ovary. Specifically, this study was designed to test the hypothesis that MXC works through the AHR to inhibit follicle growth and induce atresia.

5.3 Materials and Methods

Chemicals: MXC (99% pure) was purchased from Chemservice (West Chester, PA). Stock solutions of MXC for in vitro experiments were prepared using dimethylsulfoxide (DMSO) (Sigma, St. Louis MO) as a solvent, and in various concentrations (1.33, 13.33, and 133.33 mg/ml) that permitted equal volume of solvent to be added to individual culture wells for each treatment group. Thus, final concentrations of MXC in culture were 1, 10, and 100 μg/ml (ppm). For controls and MXC treatment groups, DMSO was used at 0.075%, which is able to solubilize MXC in aqueous media without exerting toxicity to follicles. These doses were selected for in vitro studies based on previously published studies showing that these concentrations of MXC induce toxicity in antral follicles and granulosa cell culture models (Gupta *et al*., 2006; Miller *et al*., 2005). These concentrations are relevant to occupational exposure levels. The FDA monitored the chemical contaminants in food products in the United States and calculated the average daily intake of methoxychlor in adults was up to 4 ng/kg/day (ATSDR, 2002). Normally, serum levels were found to be below the level of detection. However, a study involving an occupational exposure in farm workers showed that MXC concentrations in serum could reach as high as 5.16 μg/ml (ATSDR, 2002). Thus, the occupational exposure dose is much higher than normal human exposure and lies between the doses used in the present experiments: MXC 1 μg/ml and MXC 10 μg/ml. DMSO, ITS (insulin, transferrin, selenium), penicillin, and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). Alpha-minimal essential media (α-MEM) was obtained from Invitrogen (Carlsbad, CA). Human recombinant follicle

stimulating hormone (rFSH) was obtained from Dr. A.F. Parlow from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

Animals: Adult female cycling C57BL/6 female wild-type (WT) and AHR homozygous null (AHRKO) mice were housed in the core animal facility located at College of Veterinary Medicine, University of Illinois and maintained on 12L: 12D cycles. Mice were given *ad libitum* food and water, and temperature was maintained at 22±1 °C. Animals were euthanized at 30-35 days of age by carbon dioxide (CO_2) inhalation followed by cervical dislocation. The ovaries were removed and antral follicles were isolated as explained below. The University of Illinois Institutional Animal Care and Use Committee (IACUC) approved all protocols involving animal care, euthanasia, and tissue collection.

Screening/genotyping AHRKO and WT mice: Mice were genotyped using polymerase chain reaction (PCR) - based assays. Briefly, ear punch tissues from pups were lysed in 25 µl of buffer (1M Tris pH 8.0, 5M NaCl, 0.5M EDTA, and 20% SDS) containing 2 µl of 20 mg/ml proteinase K (Qiagen Inc.,Valencia, CA). Digestion was carried out at 55°C for 1h followed by enzyme inactivation at 100°C for 3 min. Molecular grade water (73 µl) then was added to the lysate and this mixture was subjected to PCR using primers (1) Neo F: 5'- TTGGGTGGAGAGGCTATTCG - 3 ' and (2) Neo R: 5'- CCATTTTCCACC ATGATATTCG - 3', which detect the insert in the AHR gene and primers (3) F: 5'- TCTTGGGCTCGATCT TGTGTCA - 3' and (4) R: 5'- TTGACTTAATTCCTTCAGCGG - 3', which detect the AHR gene.

The conditions for PCR were 94°C for 2 min of initial denaturation followed by 40 cycles at 94°C for 45 s, 55°C for 1 min, and 72°C for 3 min. PCR products then were subjected to agarose gel electrophoresis. The presence of a 672-bp fragment indicated that the mice were WT, the presence of 580-bp band indicated that the mice were AHRKO, and presence of both 673- and 580-bp bands indicated that mice were heterozygotes. Only WT and AHRKO homozygous mice were used in these experiments.

Granulosa cell culture and Cignal reporter assay: Ovaries from 30-35 day old mice were collected and cleaned in petri dishes containing collection media (α-MEM media, 10% FBS, 200 U/ml penicillin, 200 mg/ml streptomycin). They were then transferred to petri dishes containing supplemented media and were incubated for 20 - 30 min at 37^{o}C. Supplemented media were prepared as previously described (Basavarajappa et al., 2011). Granulosa cells (GC) were collected by puncturing follicles using syringe needles. GCs were counted using a hemocytometer chamber, and approximately 20,000 GCs were added to each well of a 96 well microplate and incubated at 37^{o}C for 3 days with media changed daily.

After 3 days of culture, when the cells reached 80-90 % confluence, GCs were transfected with negative control, positive control or xenobiotic response element (XRE) reporter plasmids (Cignal XRE reporter (luc) kit: SABiosciences) using Lipofectamine2000 (Invitrogen). Transfection was carried out according to manufacturer instructions for a transfection time of 18 h. After the transfection, medium was replaced with supplemented medium containing either DMSO or MXC (1, 10, and 100 μg/ml) or the positive control TCDD (10 nM) and incubated for 18 h. DMSO or MXC treatments were also added to negative controls to determine the specific effects and background reporter activity. The Dual-Glo Luciferase Reagent (Promega) was added

directly to medium to measure both firefly and renilla luminescences. TCDD was used as a positive control for induction of XREs because several studies indicate it binds the AHR with high affinity (Fisher et al., 1990; Poland et al., 1976). No-treatment controls were used as a control for culture conditions. XRE reporter plasmids contain a mixture of inducible AHR-responsive firefly luciferase construct and constitutively expressing Renilla luciferase construct (40:1). Negative control contained a mixture of non-inducible firefly luciferase construct and constitutively expressing Renilla luciferase construct (40:1). Positive controls contained a mixture of constitutively expressing green fluorescence protein, constitutively expressing firefly luciferase, and constitutively expressing Renilla luciferase constructs (40:1:1). Renilla luciferase constructs acted as an internal control within each well.

Antral follicle culture: Antral follicles were isolated from ovaries of WT and AHRKO mice between 30-35 days old because this is the age at which mice are cycling young adults (Basavarajappa *et al*., 2011; Gupta *et al*., 2009). Antral follicles were isolated mechanically from the ovaries based on appearance and relative size (diameter 200-400 μm) and interstitial tissue was removed using fine watch-maker forceps. About 2-3 mice were used per experiment and they yielded approximately 30-40 follicles per mouse. Once follicles were isolated, they were placed in each well in 96 well culture plates containing 150 μl of unsupplemented medium. A dose response regimen of MXC (1-100 μg/ml) and vehicle control (DMSO) was individually prepared in supplemented α-MEM. The unsupplemented medium was replaced with supplemented medium containing either DMSO or MXC (1, 10, and 100 μg/ml). Non-treated controls were used in each experiment as a control for culture conditions. The unsupplemented medium was removed slowly so that follicles could attach to the bottom of wells. Each treatment

group in an experiment consisted of 10-15 follicles.

Follicles were cultured in supplemented medium according to protocol as previously described (Basavarajappa *et al*., 2011). Follicles then were incubated for 168 hours (h) with medium changed at 96 h. At the end of 168 h, media were collected and stored at -80^{o}C for later use. In addition, some follicles were fixed in Dietrick's solution for histological evaluation of atresia and some follicles were collected, snap frozen, and stored at -80^{o}C for quantitative real time PCR as described below. Follicle cultures were repeated three times to obtain enough power for statistical analysis.

Analysis of follicle growth: WT and AHRKO antral follicles were cultured as described above for 168 h. Follicle growth was examined in 24 h intervals by measuring follicle diameters across perpendicular axes with an inverted microscope equipped with a calibrated ocular micrometer. Antral follicles were considered as those having diameters of 200 µm or greater (Miller *et al*., 2006), which correlates with the histological appearance of these follicles. Follicle diameter measurements were averaged among treatment groups and plotted to compare the effects of chemical treatments on growth over time.

Histological evaluation of atresia: The follicles collected in Dietrick's solution were processed through series of washes with ethanol: 70% for 10 min, 85% for 10 min, 95% for 7 min (2X), and 100% for 7 min (2X). They were then embedded in plastic blocks using Technovit 7100 kits from Heraeus Kulzer GmbH, Germany. The follicles were incubated in a pre-infiltration medium for one hour and then with infiltration medium overnight before they were embedded in plastic blocks using embedding medium. Pre-infiltration solution was prepared with one part 100%

ethanol : one part base solution and infiltration solution was prepared with 1 gm of hardener-1 with 100 ml of base solution. Embedding medium was prepared by adding 1 ml of hardener-2 to 15 ml of infiltration solution.

The follicles embedded in plastic blocks were sectioned into 2 μm thin sections using a microtome. Follicle sections were stained with Lee's methylene blue-basic fuchsin stain for 30 sec and washed with distilled water before they were cover slipped. Each follicle section was examined for level of atresia (follicle death) as evidenced by the presence of apoptotic bodies and reported at the highest level observed throughout the tissue. Specifically, follicles were rated on a scale of 1-4 for the presence of apoptotic bodies: 1= healthy, 2 = less than 10% apoptotic bodies (early atresia), 3 = 11-30% apoptotic bodies (mid atresia), 4 = greater than 30% apoptotic bodies (late atresia), as previously described by Miller et al. (2005) (Miller *et al*., 2005). Ratings were averaged and plotted to compare the effect of chemical treatments on atresia levels.

Hormone measurements: The media samples from at least 10-15 individual wells distributed equally across 3-4 experiments were randomly selected and subjected to enzyme-linked immunosorbent assays (ELISA) as described previously (Craig *et al*., 2010). Specifically, estradiol (E_2) levels were measured in the media using kits from DRG International (Mountainside, NJ). The sensitivity of the ELISA was 9.714 pg/ml for E_2. The intra-assay coefficient of variation (CV) was 4.7% and the inter-assay CV was 7.8%. The cross reactivity with other hormones for each type of kit was negligible.

Statistical analysis: All data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). For all comparisons, statistical significance was assigned at $p \leq 0.05$. Comparisons between

DMSO and the different doses of MXC were conducted on data obtained from 3 to 4 separate experiments using one-way analysis of variance (ANOVA) or Kruskal-Wallis test followed by Tukey's post hoc test or Mann-Whitney test or a test for linear regression when applicable.

5.4 Results

Effect of MXC on XRE reporter activity

MXC has been shown to bind to the AHR in liver cells, but it was unknown whether MXC also binds to the AHR in ovarian cells. Hence, we conducted transfection studies using XRE based reporter plasmids in granulosa cells. We found that MXC (10 and 100 µg/ml) significantly induces XRE reporter activity. The MXC-induction of XRE reporter activity was similar to that induced by the positive control TCDD (Figure 5.1).

Effect of MXC on WT and AHRKO follicle growth

Given our finding that MXC binds to the AHR, we next determined if MXC works through the AHR to inhibit follicle growth. Previous studies have shown that MXC inhibits growth of mouse antral follicles by 96 h of culture (Basavarajappa *et al*., 2011; Miller *et al*., 2005). In the present study, we evaluated the effects of MXC on growth of WT and AHRKO follicles through 168 h of culture. Evaluation of growth in WT follicles showed that MXC at 10 and 100 µg/ml significantly inhibited follicle growth compared to vehicle control (DMSO) at 96-168 h. MXC at 1 µg/ml did not inhibit follicle growth at any time point (Figure 5.2). Interestingly, evaluation of growth in AHRKO follicles showed that only MXC 100 µg/ml inhibited follicle growth compared to DMSO. MXC at 1 and 10 µg/ml did not inhibit follicle growth and thus, AHRKO follicles grew similar to controls at these concentrations of MXC.

Further, MXC inhibited follicle growth at an earlier time point in WT follicles compared to AHRKO follicles. Specifically, MXC 100 μg/ml inhibited growth at 96 h in WT follicles, but not until 120 h in AHRKO follicles (Figure 5.3).

Effect of MXC on WT and AHRKO follicle atresia

Since MXC is also known to induce atresia of antral follicles in vivo and in vitro, we next examined if it does so via an AHR pathway. In WT follicles, MXC at 10 and 100 μg/ml significantly increased atresia compared to DMSO. However, MXC at 1 μg/ml did not increase atresia compared to control. In AHRKO follicles, only MXC at 100 μg/ml significantly increased atresia compared to DMSO. MXC at 1 μg/ml did not increase atresia compared to control (Figure 5.4). When we compared atresia in treated and control follicles across WT and AHRKO genotypes, MXC at 10 μg/ml significantly increased atresia in WT, but not AHRKO follicles (Figure 5.4).

Effect of MXC on estradiol levels in WT and AHRKO follicles

MXC is also known to inhibit E_2 levels. Further, normal levels of E_2 are thought to help regulate normal follicle growth and atresia. Thus, we examined whether MXC decreases E_2 levels via the AHR. MXC significantly decreased estradiol levels in WT and AHRKO follicle types at the MXC 10 and 100 μg/ml doses, but not the MXC 1 μg/ml dose compared to DMSO (Figure 5.5).

5.5 Discussion

The current studies were conducted to test the hypothesis that MXC inhibits follicle growth and induces atresia through the AHR pathway in mouse ovarian antral follicles. Our data indicate that MXC binds to AHR in ovarian cells and that it may work through the AHR to regulate the effects of MXC on follicle growth and atresia, but not estradiol levels.

In cells, upon ligand binding, the AHR is activated and translocate into nucleus, where it forms a complex with aryl hydrocarbon nuclear translocator (ARNT). The liganded AHR-ARNT complex then acts as a transcriptional activator by binding to XREs present in 5'-flanking region of numerous genes including Cyp1a1 and Cyp1b1 (Denison and Whitlock, 1995). Studies have shown that the 5'-flanking region containing AHREs is responsible for the induction of Cyp1a1 and Cyp1b1 (Okey *et al*., 1994). In the current studies, we first assessed whether MXC binds to AHR by transfecting a XRE-reporter (luc) plasmid into granulosa cells. We showed that MXC induces the XRE-reporter plasmid, suggesting that it binds to the AHR in the ovary. Our results are consistent with previous findings that MXC binds to AHR in Hepa-1c1c7 cells (Han et al., 2007). Further, our results are consistent with an in vivo study in rats showing that MXC induces CYP1A1/1A2 activities in the liver (Oropeza-Hernandez *et al*., 2003). Interestingly, our data indicate that MXC (100 μg/ml) induces the XRE-reporter plasmid to the same degree as TCDD (10nM). TCDD and other polyaromatic hydrocarbons (PAHs) are known to bind to the AHR and induce the cytochrome P450 family of genes in different tissues, including the ovary (Okey *et al*., 1984; Okey and Vella, 1982; Poland *et al*., 1976). Studies in granulosa cells also have shown that TCDD induces *Cyp1a1* and *Cyp1b1* by increasing expression of *Ahr* (Dasmahapatra *et al*., 2001).

Previous studies have shown that MXC inhibits follicle growth and induces atresia in cultured antral follicles (Miller *et al*., 2006; Gupta *et al*., 2006). Given that MXC binds the AHR, we next tested whether MXC inhibits follicle growth and induces follicular atresia via the AHR pathway. We found that deletion of AHR can rescue follicles from MXC (10 μg/ml) induced inhibition of follicle growth and MXC induced atresia. Interestingly, the rescue only occurred at the 10 μg/ml dose of MXC and not the 100 μg/ml dose of MXC.

At the 10μg/ml dose, it is possible that MXC exclusively acts through AHR pathway to inhibit follicle growth and atresia. Hence, genetic deletion of AHR can rescue the MXC inhibition of follicle growth and atresia at that dose. However, at the 100 μg/ml dose, it is possible that in addition to AHR pathways, MXC works via other pathways such as non-genomic estrogen receptor pathways and androgen receptor pathways (Miller *et al*., 2006; Ghosh *et al*., 1999; Waters *et al*., 2001). Thus, deleting only the AHR does not rescue antral follicles from inhibition of follicle growth and induction of atresia by MXC (100 μg/ml).

Our results showed that the ability to rescue follicles from MXC-induced atresia is consistent with other studies showing that manipulation of the AHR rescues ovarian cells from other chemicals. For example: Studies have shown that AHR disruption by α-napthoflavone (ANF) reduces the ability of 9,10-dimethylbenz(*a*)antracene-3,4-dihydrodiol (DMBA-DHD) to cause cell death and as a result increases the number of fetal oocytes (Matikainen *et al*., 2002; Matikainen *et al*., 2001). Another study also showed that AHR disruption by ANF reduces the effect of PAHs such as benzo(*a*)pyrene, 3-methylcholanthrene, and 7,12-dimethylbenz(*a*)anthracene (DMBA) and as a result increases the numbers of primordial and primary follicular oocytes (Mattison *et al*., 1989; Shiromizu and Mattison, 1984, Shiromizu and

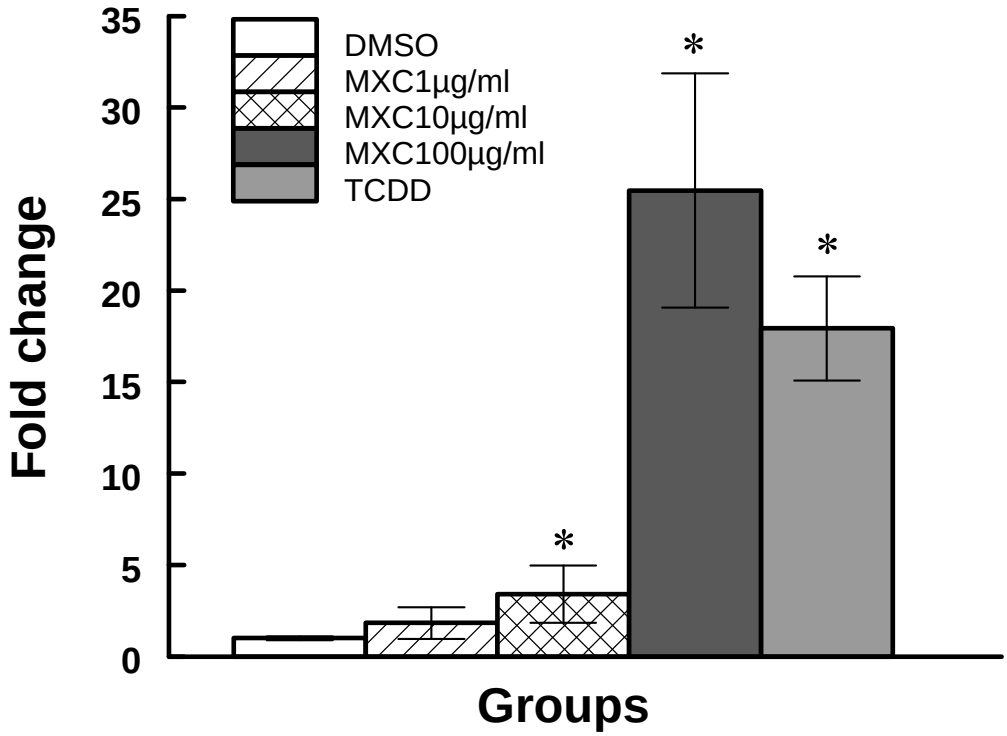
Mattison, 1985). To our knowledge this is the first study to show that genetical deletion of AHR rescues follicles from MXC induction of atresia.

Our data show that MXC (10 and 100µg/ml) decreases estradiol levels in both WT and AHRKO follicles. These results are consistent with our previous studies that showed MXC decreases E_2 levels in cultured follicles (Basavarajappa *et al*., 2011). Interestingly, we observed that AHR deletion did not rescue E_2 levels in follicles. Thus, it is evident that AHR is not involved in controlling E_2 levels in response to MXC.

In summary, these results provide mechanistic information that MXC exerts toxicity through the AHR pathway in antral follicles. The present data showed that MXC binds to AHR in granulosa cells of the ovary. In addition, current data confirmed previous data that MXC inhibits follicle growth and atresia in WT follicles. Further, we also showed that inhibition of follicle growth and induction of atresia were rescued in AHRKO compared to WT follicles. Collectively, these data show that MXC acts through the AHR pathway to inhibit follicle growth and induce atresia in antral follicles of the ovary.

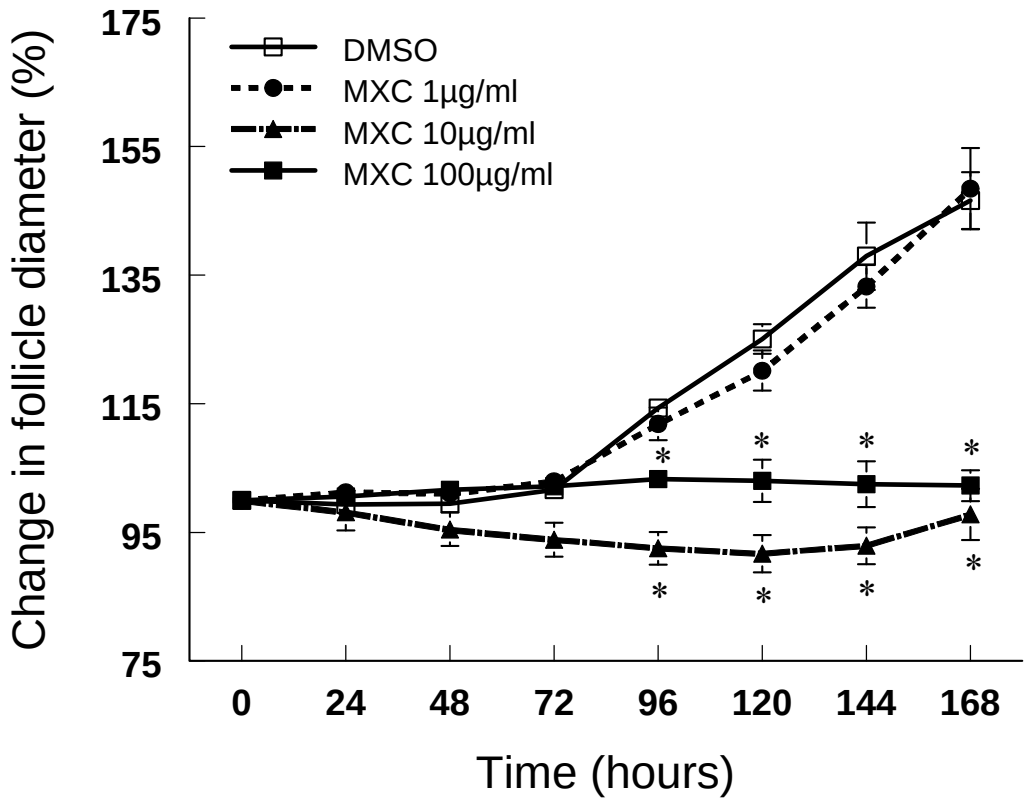
5.6 Figures and Legends

Figure 5.1 Effect of in vitro MXC exposure on XRE reporter activity



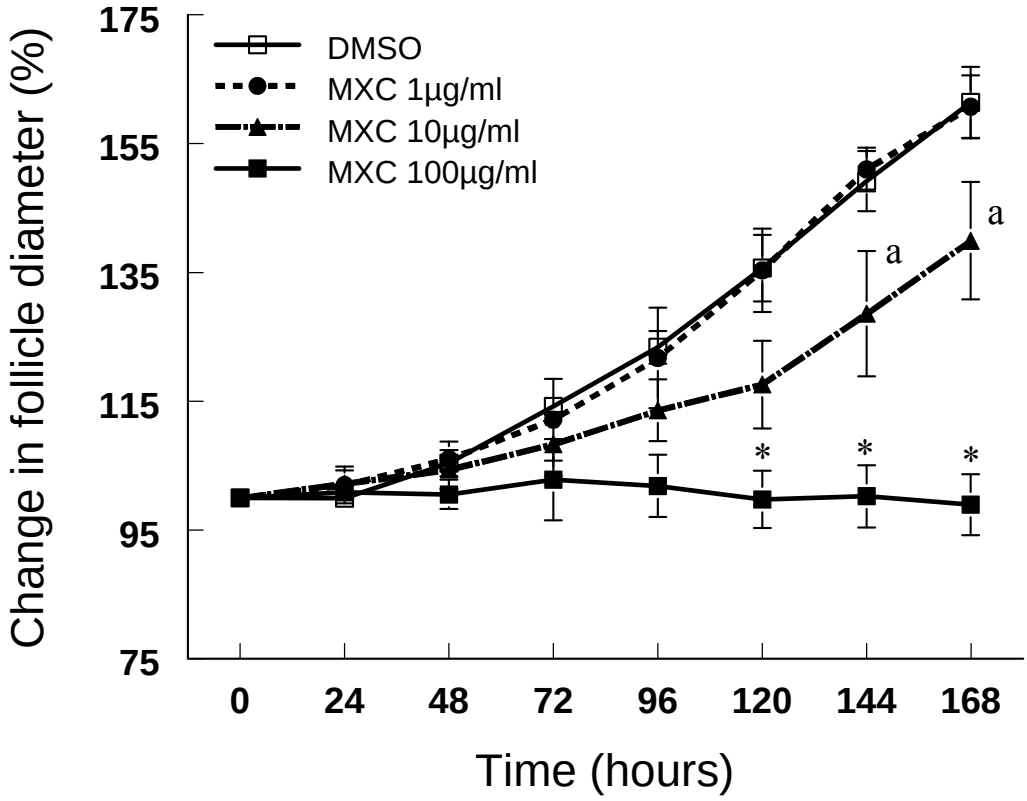
Granulosa cells (GCs) were collected from ovaries of CD-1 mice, transfected with negative or positive or XRE-reporter plasmids and then treated with vehicle (DMSO), MXC (1-100 µg/ml), or TCDD (10 nM). Dual-glo luciferase reagent was added and luminescence was measured using luminometer. Data represent means ± SE from 3 separate experiments (* indicates significant difference from vehicle controls; n=4-6 ovaries per experiment; $p \leq 0.05$).

Figure 5.2 Effect of in vitro MXC exposure on WT antral follicular growth



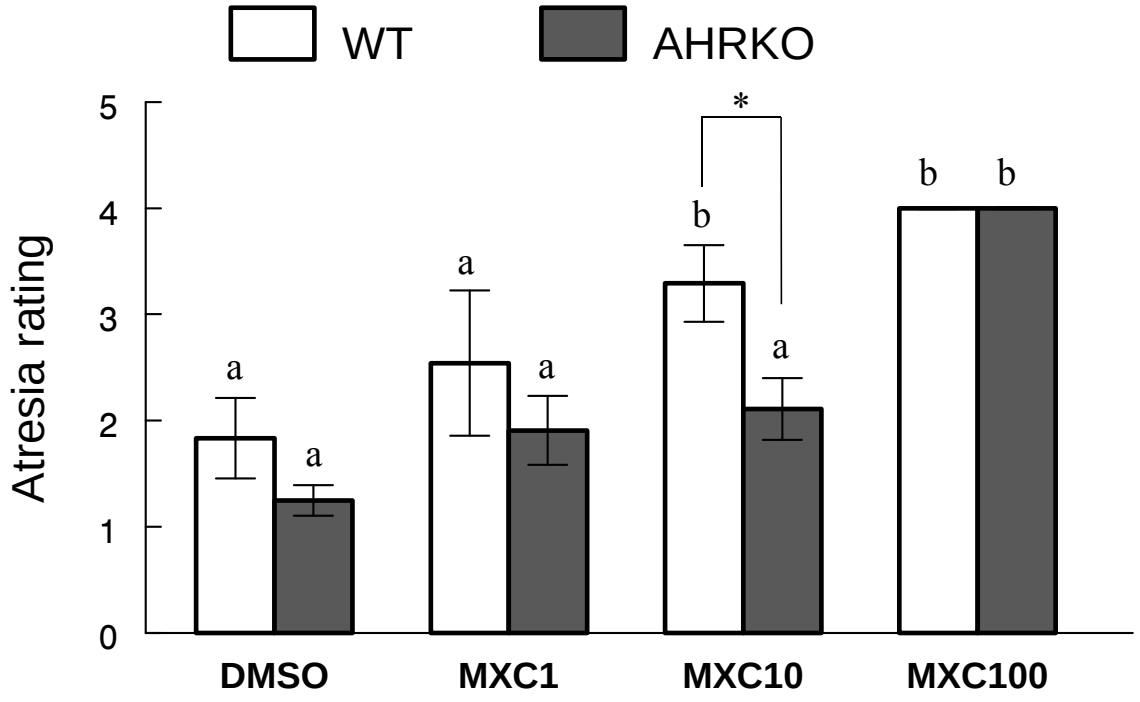
Antral follicles isolated from ovaries of WT mice were exposed to vehicle (DMSO) or MXC (1-100 µg/ml) for 168 h. Growth of follicles was monitored during culture, recorded in µm and reported as percentage change. Data represent means ± SE from 3 separate experiments (* indicates significant difference from vehicle controls; n= 12-16 follicles per treatment; $p \leq 0.05$).

Figure 5.3 Effect of in vitro MXC exposure on AHRKO antral follicular growth



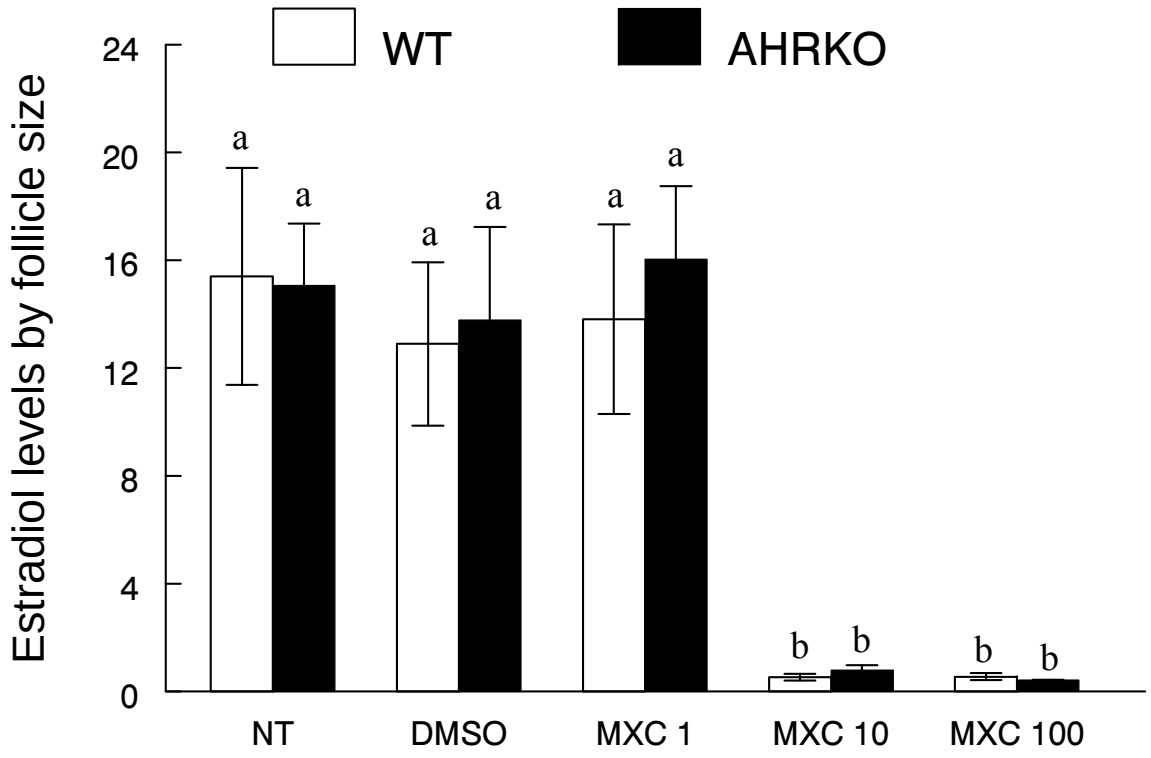
Antral follicles isolated from ovaries of AHRKO mice were exposed to vehicle (DMSO) or MXC (1-100 µg/ml) for 168 h. Growth of follicles was monitored during culture, recorded in µm and reported as percentage change. Data represent means ± SE from 3 separate experiments (* indicates significant difference from vehicle controls; **a** indicates significant difference from MXC 100 µg/ml; n= 12-16 follicles per treatment; $p \leq 0.05$).

Figure 5.4 Effect of in vitro MXC exposure on atresia of WT and AHRKO antral follicles



Antral follicles isolated from ovaries of WT or AHRKO mice were exposed to vehicle (DMSO) or MXC (1-100 μg/ml) for 168 h. At the end of culture, follicles were subjected to histological analysis of atresia. Data represent means ± SE from 3 separate experiments (* indicates significant difference between WT and AHRKO genotypes; Bars with different letters are significantly different from each other with in a genotype; n= 4 - 6 follicles per treatment; $p \leq 0.05$).

Figure 5.5 Effect of in vitro MXC exposure on estradiol (E_2) levels



Antral follicles isolated from ovaries of WT or AHRKO mice were exposed to vehicle (DMSO) or MXC (1-100 μg/ml) for 168 h. The media was subjected to measurements of E_2 levels by enzyme-linked immunosorbent assay (ELISA). Data represents means ± SEM from 3 separate experiments (Bars with different letters are significantly different from each other with in a genotype; n= 10 - 12 follicles per treatment; $p \leq 0.05$).

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Chapter VI

6.1 Summary and Conclusion

Summary

The goal of my dissertation work was to broaden our knowledge of the mechanism of action of MXC in mouse ovarian antral follicles. Understanding the mechanism of action of MXC is important as it is a well known endocrine disrupting chemical (EDC), which affects fertility in different animal models and poses risks for humans as well as wild-life. Its endocrine disrupting capability decreases ovulation and decreases birth of live fetuses (Swartz and Eroschenko, 1998). MXC targets the ovary by causing ovarian atrophy, specifically targeting the antral follicles in the ovary (Eroschenko *et al*., 1995, 1997; Borgeest *et al*., 2002). MXC decreases the number of healthy antral follicles and increases the number of atretic follicles in the female mouse ovary. MXC also inhibits antral follicle growth and induces atresia in cultured antral follicles (Miller *et al*., 2005; Borgeest *et al*., 2002, 2004). These effects of MXC destroy antral follicles and increase the rate at which follicles are depleted from the ovary. Given the role of antral follicles in production of E_2 for the physiological functions of the body, targeting follicles may increase the risks for other chronic diseases such as osteoporosis, early menopause, and reproductive senescence in humans or wildlife. Hence, the present study helps us to better understand the endocrine disruption mechanism of MXC in antral follicles by examining the effects of MXC on steroid levels, *Bcl2* factors, and by determining if MXC exerts toxicity through an AHR pathway. Collectively, I found that MXC decreases steroid levels, alters *Bcl2* factors, and involves AHR pathway during its effects on antral follicles.

In chapter III, I hypothesized that MXC decreases sex steroid hormone levels in antral follicles. In addition, I also tested the mechanism by which MXC alters steroidogenesis by examining the effects of MXC on steroidogenic as well as metabolic enzymes. I found that MXC decreases E_2, testosterone, androstenedione, and progesterone hormone levels by decreasing most of the steroidogenic enzymes and increasing the metabolic enzyme *Cyp1b1*. In the liver, MXC is metabolized to 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane (MOH) and the bisphenolic compound 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane (HPTE) by cytochrome P450 (Cyp) 1a2 and 2c29 enzymes (Stresser and Kupfer, 1997; Stresser and Kupfer, 1998). However, the MXC metabolites have not been measured in follicles. Given that antral follicles express metabolic enzymes *Cyp1a2* and *Cyp2c29* (Paulose *et al*., 2011), I did not test whether the effects of MXC on steroidogenesis are mainly due to MXC itself or MXC metabolites. Thus, future experiments should determine whether the effect of MXC on steroidgoenesis is due to MXC or its metabolites. In addition, future studies should determine the effects of MXC on protein levels of steroidogenic enzymes as the mRNA levels do not always represent protein levels. The present study did not test whether the effects of MXC on steroid levels are due to availability of substrate or down regulation of enzymes. Hence, future experiments should test the steroid levels and steroidogenic enzymes by using pregnenolone or cholesterol as a substrate in the culture. Since MXC increases *Cyp1b1* in follicles, future experiments should also test whether follicles can be rescued from MXC toxicity by blocking *Cyp1b1* using α-napthoflavone.

In chapter IV, I tested the hypothesis that MXC induces atresia by altering pro-apoptotic and anti-apoptotic factors in the antral follicles of the ovary. Specifically, I examined the effects of MXC on mRNA expression of *Bcl*$_2$, *Bcl-xL*, *Bax*, *Bok*, and *Casp3* levels and caspase activity

after short and long term exposure. I found that, at 24 h, MXC mainly increases *Bax* and does not alter *Bcl2*; thus, increasing the *Bax/Bcl2* ratio which in turn increases caspase activities. These changes together led to increased atresia in antral follicles at 48 and 96 h. We also found that MXC increases *Bax* (48 and 96 h), increases *Bcl2* (96 h) and decreases in *Bax/Bcl2* ratios (96 h) at later time points. This may be an attempt by the follicles to rescue themselves from MXC-induced atresia. However, our data indicate that once MXC induces atresia at 48 h, the follicles cannot recover from atresia throughout the culture. It is likely that any increase in anti-apoptotic factor *Bcl2* cannot compensate for pro-apoptotic effects of *Bax*. However, the present study did not examine the protein levels of these factors; hence future studies should consider measuring protein levels of Bcl2 family members using western blot or immunohistochemistry. Future studies should also determine if one can rescue follicles from MXC-induced atresia by inhibiting Bax or increasing Bcl2.

In chapter V, I tested the hypothesis that the AHR pathway is involved in mediating the effects of MXC treatment on antral follicles of the mouse ovary. Specifically, this study was designed to test the hypothesis that MXC works through the AHR to inhibit follicle growth and induce atresia. I found that MXC acts through the AHR pathway, inhibiting follicle growth and atresia in follicles. Since the present study was done using AHRKO and WT follicles in vitro and not in vivo, future studies should confirm the in vitro results by dosing AHRKO and WT mice with MXC. Given that follicle growth and atresia are restored in AHRKO antral follicles, future studies also should consider examining the effects of MXC on growth and atresia in AHR overexpressing follicles.

Conclusion

A final model describing the main findings from my work and how they intergrate with previous findings on MXC is shown below (Figure 6.1).

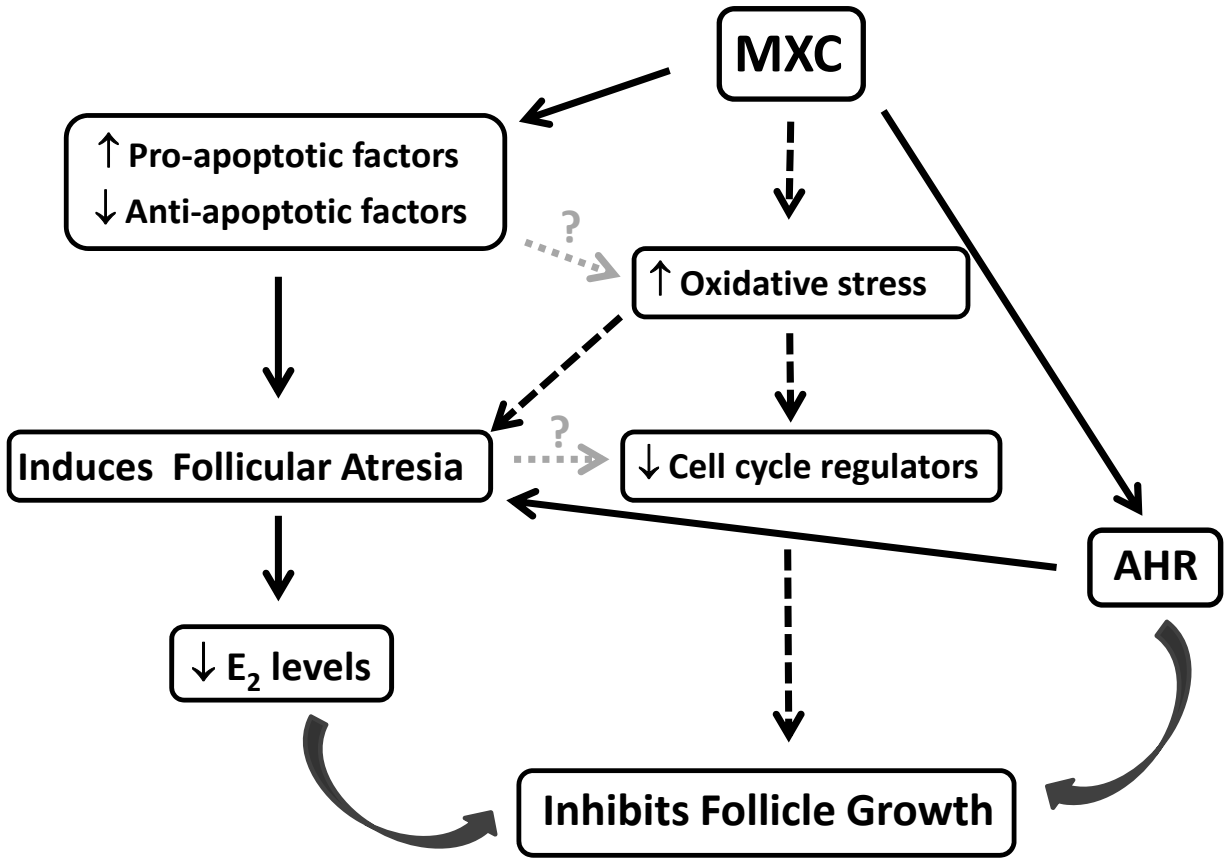
In the present study, at 24 h, MXC did not alter atresia and E_2 levels. It did, however, increase *Bax* expression without changing Bcl2 expression; thus, increasing the *Bax/Bcl2* ratio, which likely increased caspase activities. Since an increase in the *Bax/Bcl-2* ratio and an increase in caspase activity are good markers of apoptosis, I think that MXC first causes apoptosis and this eventually leads to death of follicles and as a consequence, reduced steroid levels. The mechanism by which MXC increases the *Bax/Bcl2* ratio is unknown, but it may involve the ability of MXC to induce oxidative stress. Previous studies have shown that MXC induces oxidative stress and that oxidative stress can cause apoptosis and atresia (Gupta *et al*., 2006b). It would be interesting for future studies to examine whether the increased *Bax/Bcl2* ratio precedes the production of reactive oxidative species in MXC-treated follicles. Further, it would be interesting to see if blocking the MXC-induced increase in Bax by using Bax knockout mice or Bax anti-sense protects follicles from MXC induced atresia and/or oxidative stress. By 48 h and 96 h, I observed that MXC decreases E_2 levels (MXC 1, 10, 100 μg/ml) and continues to increases atresia (MXC 1, 10, 100 μg/ml). These data suggest that the effects of MXC on steroidogenesis come after the effects of MXC on atresia and that the effects of MXC on atresia continue over time. These data are consistent with previous studies that show that MXC inhibits cell cycle regulators (Gupta *et al*., 2009). Studies indicate that inhibition of cell cycle regulators can cause cells to exit the growth cycle and enter the apoptotic pathway (Sicinski *et al*., 1996). Thus, it is possible that MXC inhibits cell cycle regulators and this pushes follicles towards atresia. Alternatively, it is possible that MXC causes atrtesia first and this leads to the decrease

in cell cycle regulator. Future studies should examine the time course by which MXC inhibits cell cycle regulators to help distinguish between these possibilities.

Finally, my work indicates that MXC may partially work through an AHR pathway because deletion of the AHR protects follicles from MXC-induced atresia and growth inhibition at the 10 μg/ml MXC dose. It is important to note, however, that AHR deletion does not protect follicles from MXC at high doses nor does it protect follicles from MXC-induced inhibition of steroidogenesis. These data suggest that MXC may work through other receptor pathways in addition to the AHR. Perhaps, at lower doses, MXC mainly works through an AHR pathway, but at higher doses, it may work through additional pathways. Since previous studies suggest that MXC may bind to estrogen and androgen receptors (Miller *et al*., 2006; Ghosh *et al*., 1999; Waters *et al*., 2001), future studies should examine whether MXC-induced atresia, inhibition of follicle growth, and reduced steroidogenesis involve estrogen receptor or androgen receptor pathways. Thus, the chemical kills follicles and inhibits steroidogenesis over time.

6.2 Figures

Figure 6.1 Final model of mechanism of action of MXC in antral follicles.



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